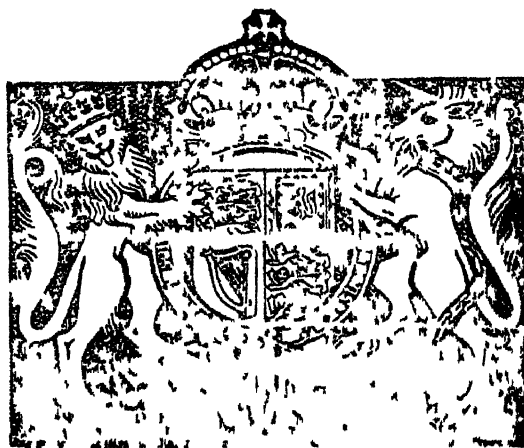


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# Studies on the Biosynthesis of Penicillin

David Bonner<sup>1</sup>

*From the School of Biological Sciences, Stanford University, California*

Received December 9, 1946

## INTRODUCTION

The investigations on elucidation of the chemical structure of penicillin have encountered many difficulties. While the probable structure is now known (4), this structure has not been confirmed by synthesis. A knowledge of the biosynthesis of penicillin would have been of material aid in work on the structure of penicillin and would be of considerable value in the production of specific penicillins. The present investigation was therefore undertaken in the hope that some knowledge concerning the biosynthesis of penicillin could be obtained.

In the few instances in which it has been possible to study extensively the biosynthesis of cellular constituents this process has been found to involve a chain of reactions. If two compounds, A and B, are normal precursors for an end product EP, the synthesis of EP might be written as  $A \rightarrow B \rightarrow EP$ . A genetic block in an organism resulting in the failure of the reaction  $B \rightarrow EP$  would give rise to a growth factor requirement for EP by this organism if EP were essential and could be supplied from the outside. In the presence of exogenous EP, B would be expected to accumulate if it were stable and not metabolized by the mutant. A genetic block resulting in the failure of the reaction  $A \rightarrow B$ , in a similar way might result in the necessity of an external supply of EP for growth, but this would result in the accumulation of A rather than B. This second type of strain would be expected to utilize B as well as EP for growth, inasmuch as the reaction  $B \rightarrow EP$  would remain

<sup>1</sup> Present address: Osborn Botanical Laboratory, Yale University, New Haven, Connecticut.

The work described in this paper was done under contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Stanford University.

unaltered. In two such strains there are therefore available both a means of producing a normal intermediate and a specific assay for it. Techniques based on this scheme have been fruitfully employed in working out several biosyntheses in *Neurospora crassa* (3, 5, 7). A genetic block in penicillin synthesis would not be expected to result in an accessory growth factor requirement since penicillin does not appear to be necessary for normal metabolism and growth. But such an interruption of any of the reactions involved in penicillin synthesis would be expected to result in the accumulation of normal penicillin

TABLE I

*Media Used*

Production medium (surface)		Production medium (submerged)	
Lactose.....	4%	Lactose.....	4%
Corn steep solids.....	4%	Corn steep solids.....	4%
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.25 g.	MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.25 g.
KH <sub>2</sub> PO <sub>4</sub> .....	0.5 g.	KH <sub>2</sub> PO <sub>4</sub> .....	0.5 g.
NaNO <sub>3</sub> .....	3.0 g.	NaNO <sub>3</sub> .....	3.0 g.
Zn (as ZnSO <sub>4</sub> ).....	0.1 g.	Zn (as ZnSO <sub>4</sub> ).....	0.01 g.
Water to make.....	1 liter	Water to make.....	1 liter
Minimal medium		Sporulation medium (after NRRL)	
NaNO <sub>3</sub> .....	3.0 g.	Glycerine.....	7.5 g.
K <sub>2</sub> HPO <sub>4</sub> .....	1.0 g.	Brer Rabbit molasses.....	7.5 g.
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.5 g.	NaCl.....	4.0 g.
KCl.....	0.5 g.	Bacto-peptone.....	5.0 g.
FeSO <sub>4</sub> .....	0.01 g.	MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.05 g.
Sucrose C.P.....	30.0 g.	KH <sub>2</sub> PO <sub>4</sub> .....	0.06 g.
Water to make.....	1 liter	Ferric tartrate.....	0.003 g.
		CuSO <sub>4</sub> .....	0.001 g.
		Agar.....	25.0 g.
		Water to make.....	1 liter

intermediates. The presence of these might then be detected by strains of a second genetic type which could convert them to penicillin.

On the basis of these considerations, a program was undertaken in which mutant strains of *Penicillium notatum* blocked in penicillin synthesis were produced. These strains have been used in studies of the biosynthesis of penicillin from the standpoint of the possible production and identification of natural precursors and in investigations of the possible use by such strains of known compounds as penicillin precursors. Although the results of these investigations are negative

it is felt worthwhile to record them so that other investigators who might be interested in using a similar approach to the problem may know of them.

Two strains, NRRL 1951.B25 and Minnesota X-1612, were used in this investigation. X-rays were used to induce mutations and treatments were carried out as described by Bonner (2). The dosages used were 100,000r units for 1951.B25, and

TABLE II  
*Morphological and Cultural Characteristics of Penicillinless Strains Derived from Penicillium notatum—NRRL 1951.B25 and X-1612*

Strain number	Morphological characteristics	pH after 7 days surface growth	pH after 5 days submerged growth	Approximate penicillin production (O.U./ml.)
75,993	Normal, no yellow exudate	7.1	7.3	0
76,209	Normal, no yellow exudate	7.8	7.6	0
76,505	Normal, no yellow exudate	7.4	7.5	0
76,509	Normal, no yellow exudate	7.0	6.8	0
76,511	Normal, no yellow exudate	5.6	5.6	0
76,530	Normal, no yellow exudate	7.2	7.0	0
77,410	Normal, no yellow exudate	6.9	6.2	0
77,459	Normal, no yellow exudate	6.9	6.5	0
77,866	Pale green	7.7	8.0	0
77,875	Normal green, slow grower	8.1	8.0	0
78,591	Dirty white, clumpy	6.5	6.2	0
78,598	Dirty white, slow grower	7.2	7.5	0
80,317	Yellow, slow grower	7.2	7.6	8
80,345	White, slow grower	6.8	6.6	5
80,397	Gray, slow grower	5.3	7.0	2
81,009	Normal, no yellow exudate	6.3	4.3	0
82,195	Dirty white, clumpy	4.1	5.1	0
82,650	Normal, no yellow exudate	6.2	6.3	0
82,605	Normal, no yellow exudate	7.9	8.9	0
82,820	Pale green, slow grower	4.4	5.5	2
83,501	Light yellow	5.0	6.7	0
83,661	Gray, lysineless	7.5	7.0	2
83,803	Normal, no yellow exudate	7.4	7.5	0
84,264	Dirty white	5.7	5.5	0
84,512	Yellow, slow grower, nicotinicless	4.2	5.9	0
85,121	Pale green	8.0	7.0	8
85,696	Dirty white	6.6	6.3	0
85,962	Normal, no yellow exudate	5.4	6.8	0
87,007	Normal, no yellow exudate	7.8	7.6	0
87,008	White, slow grower	5.0	5.5	10

TABLE II—*Continued*

Strain number	Morphological characteristics	pH after 7 days surface growth	pH after 5 days submerged growth	Approximate penicillin production (O.U./ml.)
87,277	White, slow grower requires combination of amino acids for growth	7.2	7.1	0
87,509	Pale green	7.2	6.8	0
88,053	Normal, no yellow exudate	7.5	6.6	0
88,054	Normal, no yellow exudate	7.6	7.3	0
88,809	Bluish-green	4.1	7.2	0
88,044	Pale green	7.8	6.4	5
90,512	Normal, no yellow exudate	5.3	4.2	0
90,557	Gray	5.2	6.8	0
91,009	Normal, no yellow exudate	5.2	7.3	0
91,462	Normal, no yellow exudate	7.9	8.0	0
91,627	Normal, no yellow exudate	6.2	4.2	0
91,834	Normal, no yellow exudate	7.6	8.2	0
91,210	Normal, no yellow exudate	4.9	5.5	0
91,248	Normal, no yellow exudate	4.8	5.7	0
95,311	Normal, no yellow exudate	7.3	8.1	0
95,320	Normal	7.5	7.0	8
95,327	Normal, no yellow exudate	7.7	7.7	0
95,907	Normal, no yellow exudate	7.5	8.2	0
95,924	Normal, no yellow exudate	7.5	8.2	0
96,332	Pale green	7.7	8.1	0
96,415	Normal, no yellow exudate	8.6	7.0	8
97,490	Normal, no yellow exudate	5.5	5.0	0
98,423	Normal, no yellow exudate	7.4	7.4	10
98,688	Normal, no yellow exudate	7.0	7.0	0
Normal	Yellow exudate	7.-7.5	7.-7.5	100-150

75,000r units for X-1612. Treated spores were plated out in petri dishes and genetically homogeneous colonies isolated as previously described (2).

Conidia were transferred from the single spore isolation cultures to 125 ml. Erlenmeyer flasks containing 20 ml. of production medium for surface growth (Table I). These cultures were then grown 7 days at 24°C., at which time they were assayed for penicillin production.

The culture media in which individual strains had been grown were assayed at approximately 1:8 dilution and penicillin non-producers selected by inspection. Strains identified as non-producers in this screening test were retested in both surface and submerged culture.

A total of 18,484 strains derived from NRRL 1951.B25 were tested in this manner, and 43 were retained as non-producers. A total of 5,216 strains derived from X-1612

were also tested, and 12 saved as non-producers, giving a total of 55 "penicillinless" strains derived from 23,700 isolations.

The morphological and growth characteristics of the penicillinless strains are listed in Table II. Since *P. notatum* is an imperfect fungus it is impossible to prove that these strains in all cases represent derivatives of NRRL 1951.B25, or X-1612 and are not contaminants. However, the biochemical mutants that have been isolated from these two strains following X-ray irradiation (2), and which produce penicillin, indicate that most of the morphological changes observed in the penicillinless cultures may, in other cases, be associated with simple biochemical mutations. These biochemical mutants are assumed to have been derived from *P. notatum* because they produce penicillin in abundance. Although it cannot be demonstrated directly, it is probable that the penicillinless mutant strains carry one or more mutations concerned with penicillin synthesis by the mold.

#### NATURAL PRECURSORS

On the assumption that one mutant strain might produce a substance that a second could convert to penicillin, the 55 penicillinless strains were grown in mixed culture, two at a time, in all possible combinations. These mixed cultures were grown in 125 ml. Erlenmeyer flasks containing 50 ml. of production medium for submerged growth (see Table I). The cultures were incubated 48 hours on a shaker with 96 3.5 inch strokes/minute, followed by 72 hours on a shaker with 126 2.5 inch strokes/minute. Penicillin determinations were carried out in the usual manner. Penicillin synthesis was observed in no combination.

A second technique was employed for testing natural precursor production of part of the mutants. The mutant strains were cultured individually with shaking as described above. After 5 days the culture fluid was filtered free of the mutant strain. Four-day-old shaken cultures of NRRL 1951.B25 and X-1612 were filtered and the mycelia gently washed to free it of excess medium. This washed mycelial mat was then suspended in the mutant culture filtrate and reshaken for 24 hours. Penicillin determinations were made after 2, 4, 6, and 24 hours. In no instance was penicillin production observed to be greater than that of appropriate controls.



## ACTIVITY OF KNOWN SUBSTANCES AS PENICILLIN PRECURSORS

An array of synthetic and isolated compounds of known structure has been tested individually and in combination for activity in promoting penicillin synthesis in the penicillinless strains (see Table III). These compounds were tested for the most part in submerged culture on shaking machines as described above. However, some of the compounds were tested in surface cultures and some of them in both surface and submerged culture. Compounds of questionable stability were sterilized by solution in alcohol, or by filtration in an aqueous solution through a bacterial filter. Aliquots of these were added aseptically to

TABLE III

*Compounds Tested for Penicillin Precursor Activity*

(Tests made in submerged culture at concentrations of 100 mg./l., and 10 mg./l.)  
Singly:

DL-Penicillamine  
DL-Alanine  
DL-Serine  
DL-Valine  
L(-)-Cystine  
DL-Methionine  
Phenylacetic acid  
Phenylacetamide  
Phenaceturic acid  
Ethyl phenaceturate  
*p*-Hydroxyphenylglycine  
Phenylacetyl-DL-alanine  
Phenylacetyl-DL-valine  
Phenylacetyl-DL-serine  
Phenylacetyl-DL-penicillamine  
Phenylacetylglucylvaline  
Phenylacetylthylenodiamine  
4-Carboxythiazolidine  
Thiazolidine, 2-methyl-2- $\alpha$ -isobutyric acid- $\beta$ -lactam (50 mg./l.)  
2-(Carboethoxyaminocarboethoxymethyl)-5,5-dimethylthiazolidine-4-carboxylic acid  
Methyl- $\alpha$ -formylmethylamino-4-carboxy-5,5-dimethyl-2-thiazolidine acetate  
HCl  
Benzylpenaldic acid diethylacetal  
2-Phenyl-5-ethoxyoxazole  
2-Methylthiazolidine  
N-acetyl-DL-valine  
Benzylpenicilloic acid  
Phenylacetyl-glycineamide

TABLE III—*Continued*

## In combination:

Phenylacetic acid + DL-alanine  
Phenylacetic acid + DL-penicillamine  
Phenylacetic acid + DL-valine  
DL-Penicillamine + phenylacetylglucineamide  
DL-Penicillamine + phenylacetylglucylvaline  
DL-Penicillamine + phenylacetylthylenediamine  
DL-Penicillamine + phenylacetyl-DL-alanine  
DL-Penicillamine + phenylacetyl-DL-valine  
DL-Penicillamine + DL-serine  
DL-Penicillamine + DL-serine + phenylacetic acid

## Compounds tested in surface culture, at a concentration of 1 mg./20 cc.:

Methyl benzylpenillonnate  
Benzylpenillic acid  
Desthiobenzylpenicillin  
Phenylacetyl-L-alanyl-D-valine  
Benzylpenilloic acid

## Crude degradation mixtures of commercial penicillin (activity of material used was 900 O.U./mg.). Tested over range of 1–100 mg. starting material/50 ml. medium:

Raney nickel-treated penicillin  
Methyl alcohol-treated penicillin  
Dilute HCl-treated penicillin  
Heat-treated penicillin (autoclaved 30 min. at 15 lb., pH 4.5)

sterile medium. Stable compounds were sterilized by autoclaving. In a few tests, penicillin formation was found in initial tests. After further checking, however, no instance has been verified in which penicillin production was brought about by the addition of any of the compounds listed in Table III, added singly or in combination.

## RELATION OF BIOCHEMICAL MUTATIONS TO PENICILLIN PRODUCTION

As described elsewhere (2), a large number of strains of *P. notatum* have been isolated, following X-ray or ultraviolet irradiation, with nutritional requirements different from those of the original strain. The individual requirements of these mutants include the vitamins, thiamine, biotin, choline, inositol, niacin, *p*-aminobenzoic acid and pyridoxin; the purine, adenine; the amino acids, arginine, cystine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, and tryptophan; and reduced nitrogen. In addition, several strains require unrecognized factors present in yeast extract.

All of these strains with nutritional deficiencies were checked for penicillin production. Strains requiring lysine are the only ones which show defective penicillin synthesis. About 25% of the strains requiring added lysine are unable to synthesize penicillin. This might be taken to indicate that lysine and penicillin have a precursor in common. The nature of such a precursor is unknown, since, as in the case of penicillin, little is known about the biosynthesis of lysine. A lysineless, penicillinless strain was included in all tests discussed earlier in this paper.

Methionine or cystine might be expected to serve as a precursor of penicillin, or as a precursor of penicillamine, which could in turn serve as a precursor of penicillin. On this assumption, strains requiring methionine, or methionine and cystine, were grown on amounts of methionine or cystine suboptimal for growth, and penicillin production measured in the culture medium. Penicillin was produced even at concentrations of methionine or cystine permitting only scant growth. Similar results were obtained in experiments carried out with other biochemical mutants.

#### ACKNOWLEDGMENTS

The author wishes to thank Professor G. W. Beadle for many contributions to the planning of the work reported, and the following for invaluable assistance in performing the work: Mrs. Miriam Bonner, Miss Charlotte Brown, Miss Ruth Dryer, Mrs. Nancy Lawrence, Miss Timona Miller and Miss Evelyn Vipham.

The compounds used in this work were generously supplied by Cutter Laboratories, Berkeley, Cal.; Eli Lilly and Company, Indianapolis, Ind.; Merck and Company, Rahway, N. J.; and Shell Development, Emeryville, Cal.

#### SUMMARY

Fifty-five strains of *Penicillium notatum* were produced by means of X-ray irradiation which are incapable of producing penicillin or which produce less than 5-10 units/cc. As a test for the formation of natural penicillin precursors these strains were grown in mixed culture, in all combinations of two at a time. In no culture was penicillin production observed. Various compounds, which might serve as precursors of penicillin were tested for penicillin-production activity with these penicillin-deficient strains. No compound or combination of compounds was found active. The relation of biochemical mutations to penicillin production was investigated but no significant correlation found.

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# The Enzymatic Inactivation of Indoleacetic Acid

## I. Some Characteristics of the Enzyme Contained in Pea Seedlings

Yu Wei Tang and James Bonner

From the William G. Kerckhoff Laboratories of the Biological Sciences,  
California Institute of Technology, Pasadena

Received December 9, 1946

### INTRODUCTION

During the course of early work with the plant auxins it was discovered that the active principles are readily destroyed *in vitro*, especially by oxidizing agents such as  $H_2O_2$  or potassium permanganate. The same sensitivity to oxidation is now known to characterize indoleacetic acid, the best known native plant auxin.

In the course of his experiments on the extraction of auxins from plant tissues Thimann (1) found that, when auxin solutions are incubated with leaf extracts of *Vicia faba* or of *Helianthus*, considerable losses in auxin activity ensued. He suggested that this might be due to enzymatic destruction of the auxin and called attention to the fact that leaves of *Vicia* and *Helianthus* contain polyphenol oxidase, whereas those of *Malva*, which were less active in causing auxin disappearance, are, according to Onslow (2), devoid of this enzyme. Thimann did not, however, demonstrate that the auxin disappearance which he observed was in fact enzymatic. Suggestions as to possible important physiological roles of auxin destruction *in vivo* were made by Van Overbeek (3) and De Haan and Gorter (4). Van Overbeek found that the tissues of dwarf strains of corn, such as *nana*, destroy auxin at a more rapid rate than comparable tissues of normal corn plants and that the tissues of *nana* plants are correspondingly lower in auxin content than those of the normal plants. Van Overbeek suggested that auxin destruction by corn tissues may be enzymatic and showed that, in certain instances, peroxidase activity parallels the auxin-destroying power of corn tissues. Van Overbeek, like Thimann, did not actually demonstrate the nature of enzymatic auxin inactivation. De Haan and Gorter (4) similarly suggested the possibility that differences in auxin destruction might be related to differences in stem growth rate in pea varieties. Auxin destruction by living tissues has been studied in roots and other organs (5, 6, 7, 8) by the same general technique as that used by Van Overbeek.

Conclusive demonstration of enzymatic auxin destruction has been made only by Larsen (9, 10), who has described an "auxin inactivating substance" obtained from the pressed juice of *Phaseolus* seedlings. This material, which is thermolabile and may

be purified by precipitation with 60% alcohol, possesses the ability to inactivate both auxin extracted from corn and indoleacetic acid. Larsen showed further that the inactivation of auxin in the presence of the enzyme takes place only in the presence of oxygen. The enzyme was not further characterized.

As this short review has indicated, the enzymatic destruction of auxin would appear to have an important physiological role in regulation of the amount of the growth substance in the plant. This paper is the first of a series concerning the biochemistry of the auxin-destroying enzymes and is concerned with an enzyme obtained from pea seedlings which carries out the oxidation of indoleacetic acid.

## MATERIALS AND METHODS

### *Preparation of the Enzyme*

All experiments reported below were made with preparations from etiolated pea seedlings. The pea seeds (var. Alaska) were soaked in water for 2 hours and sown in washed river sand contained in flats. The seeds were then allowed to germinate in complete darkness at 24°C. Epicotyls of the 7-day old seedlings were used as the source of enzyme. All of the manipulations incident to preparation of the enzyme were carried out in a cold room at 0–1°C. The epicotyls were ground in a blender in a minimum volume of distilled water and the juice filtered free of cellular debris. This crude juice, which will be referred to as whole cytoplasm, was either used directly or was lyophilized and stored for later use. For certain experiments the enzyme was purified as follows. The lyophilized whole juice was dialyzed against distilled water. A precipitate appeared which contained the enzyme while the soluble portion was inactive. The insoluble portion was suspended in buffer pH 6.6, centrifuged, and the clear solution again lyophilized. The resultant preparation, which is enriched 1000 times over the original whole juice or approximately 40 times over the original whole cytoplasm, appeared to be fully stable and to remain active over long periods.

### *Determination of Indoleacetic Acid*

Preliminary experiments were performed in which destruction of indoleacetic acid in reaction mixtures was followed by the *Avena* test, using the standard techniques (Went and Thimann, 11). For further work, use was made of a colorimetric determination of indoleacetic acid based on the observation of Salkowski that small amounts of indoleacetic acid form an intense red color with  $\text{FeCl}_3$  in the presence of concentrated mineral acid. This determination has been investigated in detail by Mitchell and Brunstetter (12) who were, however, unable to obtain a direct relation between amount of indoleacetic acid and color intensity over any wide range of concentrations. The method used in the present investigation is as follows. Two cc. of aqueous solution containing 5–100  $\gamma$  indoleacetic acid are pipetted into a test tube containing 8 cc. of indoleacetic acid reagent.

*Indoleacetic acid reagent:* 15 cc. of 0.5 M  $\text{FeCl}_3$ ,  
500 cc. distilled water,  
300 cc.  $\text{H}_2\text{SO}_4$ , sp. gr. 1.84.

The reagent is stable and keeps indefinitely.

Since the color develops to a maximum and then slowly fades out, it is necessary to choose a standard development time, for which 30 minutes has proven satisfactory. At the end of this time the light absorption by the solution is determined. The present experiments were made with a Klett-Summerson colorimeter using a green filter. Fig. 1 shows that a nearly linear relation holds between colorimeter reading and amount of indoleacetic acid/2 cc. aliquot over a wide range of concentrations. The indoleacetic acid determination used here appears to be relatively specific to indoleacetic acid itself. Tryptophan, indolepropionic acid, indolebutyric acid, indolecarboxylic acid, indolepyruvic acid, and indole do not give an appreciable reaction over the range of concentrations given in Fig. 1. The amide of indoleacetic acid responds as does indoleacetic acid itself.

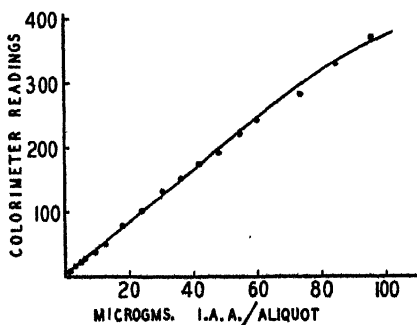


FIG. 1. Calibration Curve for Determination of Indoleacetic Acid with  $\text{FeCl}_3$  in Acid Solution.  $\gamma$  Indoleacetic acid/2 cc. aliquot as a function of reading in the Klett-Summerson colorimeter.

### Buffer Solutions

Mellvaine's phosphate-citrate buffers were used throughout for buffering the reaction mixtures. This buffer varies from 0.1 *M* at approximately pH 2 to approximately 0.2 *M* at pH 8. The stock buffer solution was diluted 4 times in the final reaction mixture.

## RESULTS

### *Existence of an Indoleacetic Acid-Destroying Enzyme*

Whole cytoplasm prepared from etiolated pea epicotyls as described above rapidly inactivates indoleacetic acid, as may be shown by following the amount of residual auxin activity with the *Avena* test.

Fifteen cc. of whole cytoplasm were mixed with 15 cc. of phosphate buffer (pH 6.5) containing either 0, 200, or 2000  $\gamma$  of indoleacetic acid/l. Five cc. aliquots were removed from each reaction mixture after 0, 1 and 3 hours. Each sample, as it was removed, was boiled to prevent further action of the system. Aliquots from each sample were diluted with an equal amount of 3% agar, standard *Avena* test blocks



TABLE I

*Inactivation of Indoleacetic Acid by Whole Cytoplasm of Etiolated Pea Seedlings*  
Determination of indoleacetic acid by the *Avena* test.

Composition of reaction mixture	Final conc. of indole-acetic acid	Length of incubation	Dilution reaction mixture per <i>Avena</i> test	Degrees curvature in <i>Avena</i> test	Amount indole-acetic acid in reaction mixture relative to initial	Per cent destruction of indole-acetic acid
	<i>γ/l.</i>	<i>hours</i>				
Whole juice + buffer	0	0	2×	0.7 ± 0.50	—	—
		1	2×	+0.3 ± 0.13	—	—
		3	2×	0.0 ± 0.02	—	—
Whole juice + buffer + indoleacetic acid	100	0	2×	4.4 ± 0.54	100%	0
		1	2×	1.2 ± 0.60	27	73
		3	2×	0.0 ± 0.00	0	100
Whole juice + buffer + indoleacetic acid	1000	0	10×	11.7 ± 0.87	100%	0
		1	2×	10.8 ± 0.59	18	82
		3	2×	0.23 ± 0.14	0	100

prepared, and the activity of each sample in the *Avena* test determined. Table I shows that the whole cytoplasm caused no significant *Avena* curvature.

When the preparation was incubated with indoleacetic acid, 73–82% of the activity was destroyed in 1 hour and all the activity within 3 hours.

TABLE II

*Inactivation of Indoleacetic Acid by Whole Cytoplasm of Etiolated Pea Seedlings*  
Determination of indoleacetic acid by colorimetric method.

Composition of reaction mixture	Final conc. of added indole-acetic acid	Incubation	Amt. of indole-acetic acid left in reaction mixture	Indoleacetic acid destroyed	Per cent destruction of indoleacetic acid
	<i>mg./l.</i>	<i>hours</i>	<i>mg./l.</i>	<i>mg./l.</i>	
Enzyme + buffer	0	0	0	—	—
		1	0	—	—
		3	0	—	—
Enzyme + buffer	15	0	15	0	0
		1	10	5	33
		3	6	9	60
Boiled enzyme + buffer	15	0	15	0	0
		1	15	0	0
		3	15	0	0

Inactivation of indoleacetic acid by whole cytoplasm may also be followed by the colorimetric method described above, as is shown by the experiment summarized in Table II.

In this experiment, 10 cc. of enzyme were added to a mixture containing 5 cc. of phosphate-citrate buffer, pH 6.6, and 5 cc. of indoleacetic acid solution, 60 mg./l. The final concentration of indoleacetic acid in the reaction mixture was hence 15 mg./l. Samples were again removed after incubation at 25°C. for 0, 1 and 3 hours, the reaction arrested in each sample by boiling for 5 minutes, and colorimetric indoleacetic acid determinations carried out on 2 cc. aliquots.

Table II shows that even the crude enzyme preparation is capable of inactivating as much as 5% of indoleacetic acid/cc. of reaction mixture within 1 hour. Table II also shows that the inactivation reaction does not take place with whole cytoplasm which had been previously heated to 100°C. for 5 minutes.

The experiments recorded in Tables I and II establish that etiolated pea seedlings contain an enzymatic system for the inactivation of indoleacetic acid. The inactivation may be followed either by the disappearance of the biological activity of the indoleacetic acid or by the disappearance of indoleacetic acid, as determined colorimetrically by its reaction with ferric chloride in acid solution. Because of the greater convenience and precision of the colorimetric method this technique was used in the bulk of the experiments which follow.

### *Optimum pH*

Determination of the pH optimum of the enzyme was made with phosphate-citrate buffers covering the range pH 3.0 to pH 8.0. For this experiment a 1.0% solution of lyophilized whole cytoplasm was used as the enzyme. To 10 cc. of the enzyme solution was added 5 cc. of indoleacetic acid solution (60 mg./l.) and 5 cc. of buffer of the desired pH. The final concentration of buffer was then 0.025–0.050 *M* and the final concentration of indoleacetic acid 15 mg./l. Samples were removed after incubation at 25°C. for 0, 1 and 3 hours.

In Fig. 2 are presented the results of this experiment, showing that the inactivation of indoleacetic acid by the enzyme exhibits a sharp pH optimum in the region 6.2–6.7, the optimum appearing to shift toward the alkaline side with time. Closely similar results were obtained in two further experiments.

### *Concentration of Substrate*

The relation of substrate concentration to rate of indoleacetic acid inactivation was determined in a series of experiments.

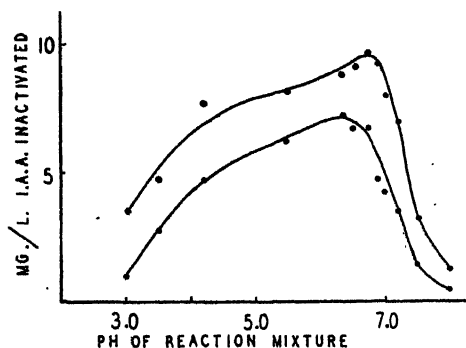


Fig. 2. Influence of pH on the Rate of Enzymatic Inactivation of Indoleacetic Acid. Lower curve after 1 hour, upper curve after 3 hours.

Lyophilized whole cytoplasm of pea epicotyls was dissolved in distilled water, 1 g./100 cc. of solution. To 10 cc. of enzyme solution was added 5 cc. of phosphate-citrate buffer pH 6.6 and 5 cc. of indoleacetic acid solution of varying concentration. Samples were removed after 0, 1 and 3 hours of incubation at 25°C.

The results of the experiment are presented in Fig. 3. The data show that the amount of indoleacetic acid inactivation is approximately a linear function of initial indoleacetic acid concentration at low concentrations, but that substrate concentration becomes non-limiting at concentrations above approximately 50 mg./l. under the present

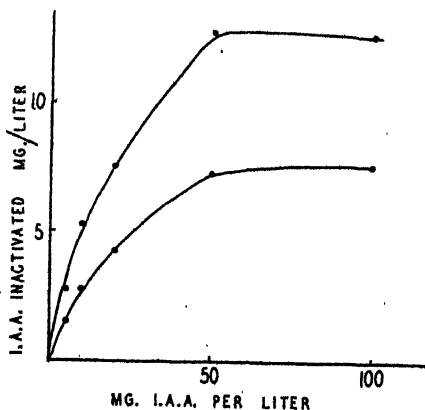


Fig. 3. Rate of Enzymatic Inactivation of Indoleacetic Acid as a Function of Substrate Concentration. Lower curve after 1 hour, upper curve after 3 hours.

conditions. It is worth noting also that at low indoleacetic acid concentrations essentially complete destruction of the added substrate took place within 3 hours. The equilibrium between indoleacetic acid and its inactivation products is, therefore, far over toward the side of inactivation.

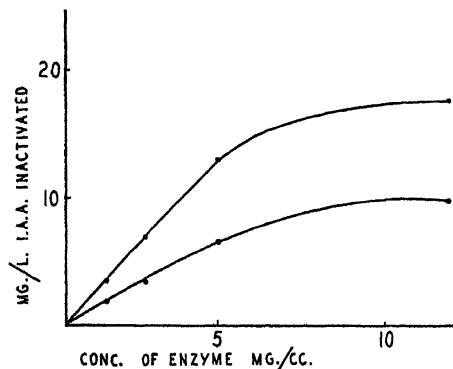


FIG. 4. Rate of Enzymatic Inactivation of Indoleacetic Acid as a Function of Enzyme Concentration. Lower curve, 1 hour; upper curve, 3 hours.

#### *Concentration of Enzyme*

The relation between rate of inactivation of indoleacetic acid and concentration of the enzyme is shown in the experiment recorded in Fig. 4.

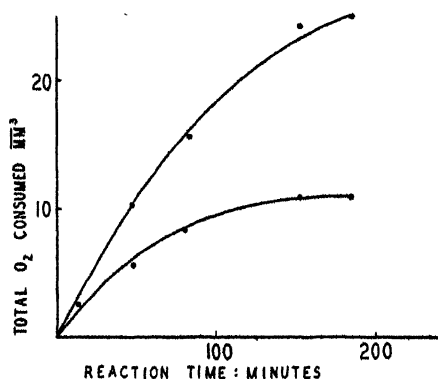


FIG. 5. Rate of Oxygen Uptake by Indoleacetic Acid Inactivation Enzyme in the Presence of Substrate. Measurements made in Warburg vessels. Upper curve: 0.33 mg. indoleacetic acid per vessel. Lower curve: 0.1 mg. indoleacetic acid per vessel.

For this experiment whole lyophilized cytoplasm was dissolved in distilled water in a series of concentrations. To 10 cc. of each solution of enzyme was added 5 cc. of phosphate-citrate buffer, pH 6.6, and 5 cc. of indoleacetic acid solution, 60  $\gamma$ /cc., so that the final concentration of indoleacetic acid was 15  $\gamma$ /cc. Samples were taken after 0, 1 and 3 hours of incubation at 25°C. and the residual indoleacetic acid determined.

Fig. 4 shows that, at low concentrations of enzyme, rate is proportional to enzyme concentration, while at higher concentrations (as can be seen in Fig. 3) substrate becomes the limiting factor.

### *Effect of Temperature*

The relation of temperature to rate of enzymatic inactivation of indoleacetic acid was not examined in detail, but data not here presented indicate that the  $Q_{10}$  over the interval 1–11°C. and 11–25°C. is of the order of 1.6. For this experiment 10 cc. of whole cytoplasm was mixed with 5 cc. of 0.1 *M* phosphate-citrate buffer and 5 cc. of indoleacetic acid solution, 60 mg./l. All reactants were brought to temperature equilibrium before mixing.

TABLE III

#### *Inhibition of the Indoleacetic Acid-Inactivating Enzyme by KCN*

All reaction mixtures contain whole lyophilized cytoplasm of etiolated pea epicotyls, 5.0 mg./cc., and phosphate-citrate buffer, pH 6.6. Time of incubation, 3 hours at 25°C.

Addition to reaction mixture	Initial concentration of indoleacetic acid	Final concentration of indoleacetic acid	Amount of indoleacetic acid inactivation	Per cent inhibition of enzymatic activity
	mg./l.	mg./l.	mg./l.	
None	15.0	8.5	6.5	—
$1 \times 10^{-6}$ KCN	15.0	8.5	6.5	0
$1 \times 10^{-4}$ KCN	15.0	11.5	3.5	46
$1 \times 10^{-3}$ KCN	15.0	13.5	1.5	77
$1 \times 10^{-2}$ KCN	15.0	15.0	0.0	100
$1 \times 10^{-1}$ KCN	15.0	15.0	0.0	100

#### *Influence of KCN on Inactivation of Indoleacetic Acid*

The action of the pea enzyme in inactivating indoleacetic acid is inhibited by low concentrations of KCN. This may be demonstrated by an experiment in which lyophilized whole cytoplasm in phosphate-citrate buffer, pH 6.6, was incubated with indoleacetic acid in the presence of various concentrations of KCN. The results are given in Table III. A concentration of KCN as low as  $1 \times 10^{-4}$  *M* caused

almost one-half inhibition in the rate of the enzymatic reaction, while higher concentrations gave correspondingly greater inhibitions. The striking sensitivity of the enzymatic system to cyanide suggests at once that a heavy metal protein may be involved. Both iron and copper proteins might be expected to form complexes with cyanide and then be inhibited. It is, however, possible to distinguish between these two possibilities on the basis of carbon monoxide, as will be shown next.

### *Inhibition of Enzyme by Carbon Monoxide*

Warburg (13) has shown that cytochrome oxidase is inhibited by carbon monoxide, but that the iron enzyme-carbon monoxide complex thus formed is stable only in the dark and is dissociated by light. Thus, the carbon monoxide inhibition of iron proteins such as cytochrome oxidase is photo-reversible (Warburg and Negelein, 14). The copper protein, polyphenol oxidase is also inhibited by carbon monoxide, but the inhibition is not photo-reversible.

Photo-reversibility of carbon monoxide inhibition has been used for discrimination between cytochrome oxidase and polyphenol oxidase in the respiration of plant tissues (Marsh and Goddard, 15). As a general discriminant between iron and copper proteins this method is unsatisfactory, since iron as well as copper proteins may fail to form complexes with carbon monoxide under ordinary conditions, *e.g.*, catalase, ascorbic acid oxidase. The indoleacetic acid-inactivating enzyme is, however, inhibited by carbon monoxide.

Carbon monoxide was generated by the action of concentrated sulfuric acid on formic acid. The washed gas was collected over water and mixed with oxygen in the proportion of 95% carbon monoxide to 5% oxygen by volume. The reaction mixtures for this experiment were placed in 50 cc. Erlenmeyer flasks provided with a low vertical septum at the bottom. Two cc. of enzyme (10 mg. whole cytoplasm/cc.) were placed on one side of the septum, while on the other side were placed 2 cc. of indoleacetic acid (30 mg./l.) in phosphate-citrate buffer, pH 6.6. The carbon monoxide-oxygen mixture was passed over the reactants for several minutes, after which the vessel was sealed off and the reactants mixed. Incubation took place at 25°C. for 1 or 3 hours, and was carried out either in total darkness or in diffuse daylight.

In Table IV are summarized the results obtained in a typical experiment. Carbon monoxide under the conditions used caused approximately half inhibition of the enzymatic activity in the dark. In the light, however, carbon monoxide had no significant influence on the rate of the reaction. This experiment, which was repeated with closely similar results, indicates strongly that the indoleacetic acid-inactivating enzyme may be an iron protein or even a heme-containing protein. It does not in any case appear to be a copper protein of the type of polyphenol oxidase.

TABLE IV

*Photo-Reversibility of the Effect of Carbon Monoxide in Inhibiting the Inactivation of Indoleacetic Acid by Pea Epicotyl Enzyme*

All reaction mixtures contained whole lyophilized cytoplasm, 5 mg./cc., indoleacetic acid, 15 mg./l. and phosphate-citrate buffer, pH 6.6.

Composition of circumambient gas	Time of incubation	Initial amount indoleacetic acid	Final amount indoleacetic acid	Indoleacetic acid inactivated	Per cent inhibition by CO
	<i>hours</i>	<i>mg./l.</i>	<i>mg./l.</i>	<i>mg./l.</i>	
Air	1	15.0	8.8	6.2	—
	3	15.0	5.0	10.0	—
95% CO+5%	1	15.0	12.0	3.0	52
O <sub>2</sub> in dark	3	15.0	10.0	5.0	50
95% CO+5%	1	15.0	9.2	5.8	6
O <sub>2</sub> in light	3	15.0	5.0	10.0	0

*The Oxidative Nature of Indoleacetic Acid Inactivation*

It has long been supposed that the *in vivo* destruction of auxin may be oxidation and Syre (16) has, in fact, shown that auxin loss does not take place in roots kept in an atmosphere of nitrogen. Larsen has shown directly in his work with the bean seedling that the presence of oxygen is essential to auxin inactivation *in vitro*. That oxygen is required for the enzymatic inactivation of indoleacetic acid by the enzyme of pea epicotyls was shown by the following experiment.

Two cc. of enzyme solution, containing 40 mg. of whole lyophilized cytoplasm/cc., was placed on one side of the septum of Erlenmeyer flasks similar to those used in the experiment of Table IV. On the other side was placed 2 cc. of solution containing phosphate-citrate buffer, pH 6.6, and indoleacetic acid, 30 mg./l. The flasks were then

TABLE V

*Dependence of Enzymatic Inactivation of Indoleacetic Acid on the Presence of Oxygen*

Experiment No. 171. Each reaction mixture contains 10 mg. whole lyophilized cytoplasm/cc. Phosphate-citrate buffer, pH 6.6.

Atmosphere over reaction mixture	Time of incubation	Initial indoleacetic acid	Final indoleacetic acid	Indoleacetic acid inactivated
	<i>hours</i>	<i>mg./l.</i>	<i>mg./l.</i>	<i>mg./l.</i>
Air	3	15.0	6.0	9.0
Argon	3	15.0	15.0	0.0
Oxygen	3	15.0	4.5	10.5

connected to gas systems and their gas spaces swept out either with air, with oxygen-free argon, or with pure oxygen. Samples of the reaction mixture were collected and analyzed for indoleacetic acid after 3 hours of incubation at 25°C.

Table V shows that indoleacetic acid inactivation was completely suppressed in the absence of O<sub>2</sub> and presence of argon. Similar results were obtained in other experiments in which the reaction vessels were merely evacuated. In the presence of pure O<sub>2</sub>, however, inactivation proceeds as vigorously as in air.

Similarly, it can be shown that oxygen is consumed during the inactivation of indoleacetic acid by the enzyme.

For this experiment, 2 cc. of dialyzed whole cytoplasmic protein were placed in the main compartment of each of a series of conical Warburg vessels, 0.2 cc. of 5% KOH was placed in the central well of each vessel, and 0.3 cc. of a solution containing the desired amount of indoleacetic acid was placed in the side arm of each vessel. The vessels were attached to their manometers, brought to temperature equilibrium at 30°C., and the contents of vessel and side arm mixed. No appreciable oxygen was consumed by the enzyme preparation in the absence of indoleacetic acid. In the presence of indoleacetic acid an increased rate of O<sub>2</sub> uptake was evident within 15 minutes, but disappeared over a period of 2.5-3 hours. (Fig. 5)

Table VI contains the detailed calculations from a similar experiment in which the amount of indoleacetic acid left in the vessels at the end of the experiment was also determined. In this experiment CO<sub>2</sub> evolution during the course of the reaction was also followed in vessels

TABLE VI

*Oxygen Consumption and CO<sub>2</sub> Production during the Enzymatic Inactivation of Indoleacetic Acid*

Reaction carried out in Warburg vessels at 30°C. Purified cytoplasmic protein as enzyme. pH 6.6. Experiment No. 187.

Vessel	Amount of indoleacetic acid in vessel		Total O <sub>2</sub> consumed	Total CO <sub>2</sub> evolved	Indoleacetic acid inactivated
	Initial	Inactivated in 3 hours			
	$\gamma$	$\gamma$	mM	mM	mM
1	None	0	—	—	—
2					
3	333	225	$1.25 \times 10^{-3}$	$1.23 \times 10^{-3}$	$1.28 \times 10^{-3}$
4					
5	100	80.5	$0.43 \times 10^{-3}$	$0.40 \times 10^{-3}$	$0.46 \times 10^{-3}$
6					



to which 0.2 cc. of 1 *N* H<sub>2</sub>SO<sub>4</sub> rather than KOH was added to the central well. At the expiration of the experimental time, bound CO<sub>2</sub> was liberated from the reaction mixture by addition of acid. Such bound CO<sub>2</sub> was, however, negligible in quantity. Table VI shows that over a period of 3 hours very nearly 1 mole of CO<sub>2</sub> was liberated per mole of O<sub>2</sub> consumed by the system. In addition, an approximate correspondence between the amount of indoleacetic acid inactivated and the evolution of CO<sub>2</sub> was found, as can be seen in Table VI. This was true at two levels of substrate concentration. Similar results were obtained in a series of further experiments.

In summary, oxygen appears to be an essential component of the enzymatic indoleacetic acid inactivation system. Approximately one molecule of O<sub>2</sub> appears to be consumed per molecule of indoleacetic acid inactivated. At the same time one molecule of CO<sub>2</sub> is liberated.

### *The Fate of Indoleacetic Acid*

It would be of interest to know what products are formed when indoleacetic acid is oxidatively inactivated by the enzyme of pea epicotyls. A pertinent observation is the fact that the indoleacetic acid nucleus remains intact. This was shown by use of the Hopkins-Cole reaction in which the indole nucleus forms a colored complex with glyoxylic acid in the presence of copper ions and concentrated H<sub>2</sub>SO<sub>4</sub>. The reaction, formation of a purple color, is given by tryptophan, indoleacetic acid, indolecarboxylic acid, indole, and other compounds containing the indole nucleus. Table VII shows that, in a given experi-

TABLE VII

#### *Persistence of the Indole Nucleus during Enzymatic Inactivation of Indoleacetic acid*

The reaction mixture contains lyophilized whole cytoplasm, 5 mg./cc., indoleacetic acid 13.0 mg./l. and phosphate-citrate buffer, pH 6.6. Incubation for 3 hours at 25°C.

Method of colorimetric determination used on reaction mixture	Amount of substance in reaction mixture:		Amount destroyed	Per cent destruction
	Initial conc. 0 time	Final conc. after 1 hour		
	mg./l.	mg./l.	mg./l.	
Ferric chloride test for indoleacetic acid	13.0	4.7	8.3	64
Hopkins-Cole test for total indole nucleus	8.7	8.7	0.0	0

ment, 64% of the indoleacetic acid was destroyed while the total indole nucleus present in the reaction mixture remained unaltered. This result, which was obtained repeatedly in other experiments, indicates that the oxidative inactivation of indoleacetic acid must be owing to alterations which occur in the side chain of the molecule. The enzymatic oxidation product of indoleacetic acid is, further, a neutral compound, since it can be removed from the reaction mixture by shaking out with ether at neutral or alkaline pH.

### *Substrate Specificity*

The indoleacetic acid-inactivating enzyme does not attack indoleacetamide, as can be shown by following the concentrations of indoleacetamide in a reaction mixture by the same ferric chloride method used for indoleacetic acid. It would appear that the free carboxyl group is essential for attack of the substrate by the enzyme. The ability

TABLE VIII

*Influence of Various Auxin Analogs on the Rate of Enzymatic Inactivation of Indoleacetic Acid*

Expts. 112 and 168. Whole cytoplasm as enzyme. Phosphate-citrate buffer pH 6.6.

Reaction mixture	Amt. of indoleacetic acid inactivated. mg./l./8 hrs.
Indoleacetic acid, 15 mg./l. Alone	10.5
Indoleacetic acid + indoleacetamide, 15 mg./l.	10.0
Indoleacetic acid + indolebutyric acid, 15 mg./l.	9.9
Indoleacetic acid + indolepropionic acid, 15 mg./l.	11.0
Indoleacetic acid + tryptophan, 15 mg./l.	10.1
Indoleacetic acid + naphthaleneacetic acid, 50 mg./l.	10.7
Indoleacetic acid + phenylacetic acid, 50 mg./l.	10.2
Indoleacetic acid + <i>cis</i> -cinnamic acid, 50 mg./l.	10.2
Indoleacetic acid + <i>trans</i> -cinnamic acid, 50 mg./l.	10.8
Indoleacetic acid + 2,6-dichlorophenoxyacetic acid, 50 mg./l.	11.0
Indoleacetic acid + naphthylacetamide, 50 mg./l.	11.0
Indoleacetic acid + naphthalenepropionic acid, 50 mg./l.	11.0

of the enzyme to attack indolebutyric acid, indolepropionic acid, and tryptophan was studied directly by following the concentration of each of these compounds in appropriate reaction mixtures by means of the Hopkins-Cole reaction, since the color intensities developed by the 3 compounds are more intense than that given by the equivalent amount of indole and indoleacetic acid. The enzyme does not attack

indolebutyric acid, indolepropionic acid, or tryptophan appreciably over a 3-hour period.

Further evidence regarding the specificity of the enzyme was obtained from experiments in which the inactivation of indoleacetic acid was studied in the presence of various auxin analogs and auxin-like compounds. It would be anticipated that, if a substance related to auxin possessed the ability of combining with the enzyme, it might compete with indoleacetic acid for position on the enzyme surface and lead to competitive inhibition of the rate of indoleacetic acid inactivation. No one of 11 auxins and related substances exerted any influence on the rate of indoleacetic acid inactivation, as is shown in Table VIII. These data together suggest that the indoleacetic acid-inactivating enzyme may be exceedingly limited in range of substrates attacked.

#### SUMMARY

1. An enzyme system which causes the inactivation of indoleacetic acid is contained in etiolated pea epicotyls. The inactivation of indoleacetic acid may be followed by decrease in biological activity in the *Avena* test or by a chemical method of indoleacetic acid determination. The enzyme, which is soluble only in dilute salt solutions, may be purified by dialysis of whole pea epicotyl cytoplasm and contains no readily dialyzable cofactor.

2. The pH optimum of the enzyme is in the range of pH 6.2-6.7.

3. Rate of inactivation of indoleacetic acid is approximately a linear function of substrate concentrations at concentrations below 20 mg. of indoleacetic acid per liter under the conditions used, but is independent of substrate concentrations at concentrations above 50 mg. per liter. Rate of indoleacetic acid inactivation is approximately proportional to enzyme concentrations at low concentrations of enzyme.

4. Enzymatic inactivation of indoleacetic acid is inhibited by KCN, one-half inhibition being given by slightly over  $1 \times 10^{-4}$  M KCN under the conditions used. The reaction is also inhibited in the dark by carbon monoxide, the inhibition being fully reversible by light.

5. Enzymatic inactivation of indoleacetic acid takes place only in the presence of O<sub>2</sub>. Approximately one molecule of O<sub>2</sub> is consumed per molecule of indoleacetic acid inactivated. One molecule of CO<sub>2</sub> is evolved per molecule of indoleacetic acid liberated.

6. The indole nucleus is not attacked during the enzymatic inactivation of indoleacetic acid. On the contrary, the side chain of the indoleacetic acid is the site of enzymatic attack.

7. The indoleacetic acid-inactivating enzyme does not attack indoleacetamide, indolebutyric acid, indolepropionic acid, indolecarboxylic acid, or tryptophan. Neither do these or other auxins and auxin-like compounds tested, 11 in all, influence the rate of inactivation of indoleacetic acid when they are present in a reaction mixture containing the latter substrate. These auxins and auxin analogs do not appear to be capable of combination with the enzyme. The indoleacetic acid-inactivating enzyme of etiolated pea epicotyls possesses a considerable degree of substrate specificity.

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# Assimilation of Acetate by Yeast

A. G. C. White and C. H. Werkman

*From the Industrial Science Research Institute, Iowa State College, Ames, Iowa*

Received November 14, 1946

## INTRODUCTION

Tracer studies of Rittenberg and Bloch (1) on fat formation and of Lifson *et al.* (2) and Lorber *et al.* (3) on carbohydrate synthesis, have indicated that the acetic acid molecule *per se* may be incorporated into the fatty or carbohydrate constituents of the animal body. The present work was undertaken to determine whether or not a similar situation obtains in plant tissues, particularly with reference to the fat constituents.

Smedley-MacLean and Hoffert (4) have shown that the incorporation of various two-carbon compounds, including acetic acid and ethyl alcohol, in an aerated medium containing metabolizing yeast gives rise to an appreciable increase in the fat content of the cells. That this increase is due to utilization of the compound added rather than at the expense of the stored carbohydrate of the yeast can be readily deduced from their analytical data. Since it appears desirable to have direct proof of synthesis, the present experiments utilize  $C^{13}$  as a tracer.

## EXPERIMENTAL

### *Methods*

The organism used in these experiments was an Iowa State stock culture of bottom yeast, *Saccharomyces cerevisiae*, which has been shown to be unable to oxidize acetate in the Warburg respirometer. The organism was stored on malt agar slants. Before each experiment the yeast was inoculated into 100 ml. of sterile medium of the following composition: Difco malt extract broth, 1%; glucose, 1%;  $K_2HPO_4$ , 0.1%; and  $NH_4Cl$ , 0.1%. After 24 hrs. at 30°C. the contents were transferred to 10 l. of a similar medium and incubated for an additional 24 hrs. Cells were harvested by means of a Sharples centrifuge and used immediately.

The procedure was developed to insure maximum production of fat since it was found that, with our particular strain of yeast, the fat content had increased greatly

during the first 24 hrs. of growth and declined rapidly thereafter. By use of the above technic consistent increases in fat of about 110% were obtained with acetate, based on the initial fat concentration of the yeast.

*Isotopic Acetate.*  $\text{CH}_3\text{C}^{13}\text{OOH}$  was prepared by the Grignard procedure and contained an excess of  $\text{C}^{13}$  of 4.33% in the carboxyl carbon.

The experiments were carried out in 1 l. cylindrical glass flasks. Twelve g. (wet wt.) of the 24 hr. yeast cells were suspended in 300 ml. of 0.025 *M* phosphate buffer containing 32 mM  $\text{CH}_3\text{C}^{13}\text{OONa}$ . The pH was adjusted to 6.8–7.0 and the suspension aerated at the rate of approximately 150 ml./min. through a sintered glass disk at the base of the flask. With each experiment containing the isotopic acetate two controls were run. One 12 g. sample of yeast was analyzed at 0 time, a second was suspended in 300 ml. of the same phosphate buffer and carried through the same procedure as the test organisms but without acetate.

Flasks were incubated at 30°C. for 46 hrs. and continuously aerated. Immediately after incubation the contents were centrifuged, the cells washed twice with water and hydrolyzed for 2 hrs. according to the procedure of Smedley-MacLean (4). The supernatant and washings from the cells were made alkaline to phenolphthalein, concentrated to approximately 50 ml., acidified, and the residual acid recovered by steam distillation. Only acetic acid was present as shown by the partition method of Osborn *et al.* (5).

The cell hydrolyzate was made alkaline to phenolphthalein and filtered hot. The precipitate was washed twice with hot distilled water and the filtrate made to 250 ml. Reducing sugar was then determined as glucose on the filtrate by the method of Stiles *et al.* (6). The precipitate from the hydrolysis was dried for 12 hrs. in a vacuum desiccator and extracted for 24 hrs. with ether in a Soxhlet extractor. The extracted fat was dried *in vacuo* and weighed. To further insure removal of any residual acetic acid, 0.5 ml. of glacial acetic acid in 10 ml. of  $\text{H}_2\text{O}$  was added to the fat to dilute any labeled acetate present so that its effect would be negligible, and a distillate equivalent to 10–12 volumes of the suspension collected by steam distillation. This procedure effectively removed all free volatile acids. The fat was once again dried *in vacuo* and an aliquot converted to  $\text{CO}_2$  by dry combustion. The  $\text{CO}_2$  was trapped in 2 *N*  $\text{CO}_2$ -free alkali solution and used for analysis on the mass spectrometer.

The residual fat was saponified with 5% alcoholic KOH for 1 hr. Alcohol was removed *in vacuo* and the residue suspended in water. The fatty acids were removed by extraction with ether and dried *in vacuo*. An aliquot was then converted to  $\text{CO}_2$  by the above procedure.

To determine the presence of the isotope in the carbohydrate fraction, the above filtrate was freed of protein with lead, excess lead removed with  $\text{H}_2\text{S}$ , and the excess  $\text{H}_2\text{S}$  removed by aeration. The glucose present in the filtrate was then converted to lactic acid by the procedure of Wood *et al.* (7) using *Lactobacillus casei*, and the lactic acid formed degraded to acetaldehyde and  $\text{CO}_2$ .

## RESULTS

The effect of acetate on the fat content of yeast cells is shown in Table I. In two typical experiments the average increase is 107% cal-

TABLE I  
*Effect of Acetate on Fat Content of Yeast Cells*

Time	Fat	Reducing sugar as glucose	Increase in fat
	mg.	mg.	per cent
0	64.0	966	
46 hrs.—no acetate	63.4	556	0
46 hrs.+acetate (isotopic)	132.7	638	109
0	74.9	1100	
46 hrs.—no acetate	76.8	267	2.5
46 hrs.+acetate	153.2	882	105

After two hrs. hydrolysis with 1 N HCl.

culated on the basis of mg. of fat formed in excess of that initially present.

Table II shows a typical set of data using the labeled acetate. The carbohydrate and fat analyses for this experiment are given in Table I, first section. Considerably more of the isotope is found in the fatty acid fraction, results which are to be expected if we assume the excess fat to be formed from the acetate added. (See discussion.)

TABLE II  
*Heavy Carbon Distribution in Yeast Cell Constituents*

Fraction analyzed	mM	C <sup>13</sup>
Residual acetic acid	0.171	0.08
Fat		1.49
Fatty acids		1.14
Lactic acid from glucose		0.09
—COOH of lactic acid		0.30
CH <sub>3</sub> — group of lactic acid		0.02
—CHOH— group of lactic acid		0.00

C<sup>13</sup> expressed as atom-% excess.  $\pm 0.02$  error of mass spectrometer.

The lactic acid derived from the glucose contains excess C<sup>13</sup> in only the carboxyl group as is to be expected if the intact acetate molecule is metabolized according to the present scheme of carbohydrate metabolism.

To show indirectly that the acetic acid was not first oxidized to CO<sub>2</sub> but was utilized directly, the following experiment was devised. Two



duplicate flasks were set up as before, one containing labeled and the other normal acetate. To the solution of the normal acetate were added 25 mM of  $\text{NaHC}^{13}\text{O}_3$  while an equivalent amount of normal  $\text{NaHCO}_3$  was added to the flask containing the labeled acetate. The flasks were then connected to the macrorespirometer of Wood *et al.* (8). Incubation and aeration were carried out as before with the exception that the system was closed and the respiratory  $\text{CO}_2$  collected in an alkali trap. Degradation and analysis were carried out as described above.

Table III shows the results of the above experiments. It will be noted that no  $\text{C}^{13}$  is present in the fat from the yeast incubated in the presence of heavy carbon bicarbonate and normal acetic acid although the increase in fat is approximately the same.

TABLE III

*Comparison of Distribution of Heavy Carbon in Yeast Between  
 $\text{CH}_3\text{C}^{13}\text{OOH} + \text{NaHCO}_3$  and  $\text{CH}_3\text{COOH} + \text{NaHC}^{13}\text{O}_3$*

	$\text{CH}_3\text{C}^{13}\text{OOH} + \text{NaHCO}_3$	$\text{CH}_3\text{COOH} + \text{NaHC}^{13}\text{O}_3$
	$\text{C}^{13}$ per cent	$\text{C}^{13}$ per cent
Residual acetic acid	0.08	0.00
Respiratory $\text{CO}_2$	1.25	0.85
Fat	0.84	0.01
Fatty acids	0.80	0.00
—COOH from lactic acid	0.15	0.09
$\text{CH}_3$ — from lactic acid	0.01	0.00
—CHOH from lactic acid	0.0	0.00

Values expressed as atom-% excess  $\text{C}^{13}$ .  $\pm 0.02$  error of mass spectrometer.

## DISCUSSION

From the calculation of the dilution of heavy carbon by the fat initially present in the yeast cells with the assumption that the  $\text{C}^{13}$  is in alternate positions in the fatty acid chain, it appears that the excess fat formed in the presence of acetic acid is derived almost entirely from the added acid. Dilution factor  $\times$  isotope concentration of acetate = isotope concentration of fat. Calculation:  $\frac{68.7 \times 2.17}{132.7}$   
 = 1.12 calc.; 1.14 found.

The conclusion appears to be in accordance with the concept that fatty acids are built up from  $C_2$ -fragments as has been shown for the lower members of the series by Barker *et al.* (9) and Medes *et al.* (10).

From the results of Smedley-MacLean and Hoffert (4), and our own experience, phosphate is apparently necessary for the formation of large increases of fat observed, although some excess fat is obtained in its absence. Whether this is caused by the formation of an energy-rich compound such as acetyl- $PO_4$  before synthesis takes place cannot be determined by the present experiments. It does seem clear, however, that a  $C_2$ -compound must be involved in some manner since these cells are apparently able to convert acetate directly into fat. Two facts appear to rule out the prior conversion to carbohydrates. First, the excess of heavy isotope in the carbohydrate fraction should have been at least as high as, if not higher than, that of the fat. Secondly (Table I), the carbohydrate content of the cells containing the acetate was greater after the period of incubation than the aerated control, which may be accounted for by an actual synthesis of carbohydrate from the added  $C_2$ -compound or as a true sparing of the cellular carbohydrate.

The results of fat synthesis in the presence of  $NaHC^{13}O_2$  provides additional evidence in support of the utilization of the intact acetate molecule. If the acetate were first decomposed by the cell to  $CO_2$  and  $H_2O$ , the  $CO_2$  from the bicarbonate would readily have exchanged with that from the acetate. That this is not the case is indicated by the lack of excess  $C^{13}$  in the fat from these cells. The carbohydrate fraction, however, did contain excess  $C^{13}$  showing that an exchange with the respiratory  $CO_2$  had taken place.

The presence of excess  $C^{13}$  in the respiratory  $CO_2$  of the cells incubated in the presence of labeled acetate can be explained on the basis of uptake of the intact acetate molecule during carbohydrate metabolism as has been shown by Lorber *et al.* (3) and Wood *et al.* (7). The position of the excess  $C^{13}$  in the glucose chain, *i.e.*, 3 and 4, is that which would be expected if the current schemes for carbohydrate metabolism are accepted. However, since the excess isotope in the respiratory  $CO_2$  is much higher than that found in the glucose molecule, one is led to assume that either another mode of breakdown is present or that a large percentage of the acetate assimilated does not find its way back to glucose.

## SUMMARY

Large increases of fat are formed by metabolizing yeast cells in the presence of sodium acetate.

By the use of the heavy isotope of carbon this increase has been shown indirectly to be caused by assimilation of the intact acetate molecule. The acetate appears to exert a "sparing" action on the cellular carbohydrate since a large decrease in stored carbohydrates occurs in the absence of the acid.

Acetic acid, labeled with  $C^{13}$  in the carboxyl group, appears to be readily incorporated into the carbohydrate metabolizing system of the cell. The isotope is found in excess in positions 3 and 4 of the glucose molecule which are the positions indicated by the present theory of carbohydrate metabolism.

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# Studies on the Sulfur Metabolism of *Escherichia coli*.

## I. The Growth Characteristics and Metabolism of a Mutant Strain Requiring Methionine

J. O. Lampen, M. J. Jones and A. B. Perkins

*From the Chemotherapy Division, Stamford Research Laboratories,  
American Cyanamid Company, Stamford, Connecticut*

Received December 18, 1946

### INTRODUCTION

Mutant strains of *E. coli* No. 15<sup>1</sup> which require the presence of some preformed molecule for growth have been obtained by X-ray treatment in this Laboratory (1). Similar changes have been induced in other strains of *E. coli* (2, 3) and in *Neurospora* (4). The nutritional requirements of these mutant cultures indicate that they vary from the parent strain only in their inability to carry out a single synthetic reaction.

The present work concerns the growth requirements and metabolism of the methionine-requiring mutant 532-171.<sup>1</sup> This strain is stable during prolonged subculture in the presence of excess methionine (1). On a complete medium it grows as rapidly as the parent strain and to a slightly greater maximum turbidity. These characteristics led us to study the suitability of this culture for the determination of methionine in order to test the possible general usefulness of these mutants for microbiological assay of amino acids.

### EXPERIMENTAL PROCEDURES

Stock cultures of the mutant were kept on agar slants of our "complete" medium (1) and transferred monthly. The organisms to be used as inocula for all experiments except the determination of growth rates were grown for 7 hours in the "complete" medium without the agar, centrifuged, washed with saline buffer (1) and resuspended in the original volume of buffer. This suspension was diluted 100-fold and one drop/10

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<sup>1</sup> *E. coli* No. 15 is available from the American Type Culture Collection as No. 9723 and mutant 532-171 as No. 9663.

ml. was used as inoculum. This procedure yields an initial cell concentration of 50,000/ml. Organisms grown in Nutrient Broth (Difco) have also proven satisfactory. However, the "complete" medium was generally used to insure cells of maximum viability. Unless otherwise indicated, all cultures were incubated at 37°C.

The basal, synthetic medium employed was that of MacLeod (5) with the following composition (per liter): NaCl, 5.0 g.;  $(\text{NH}_4)_2\text{SO}_4$ , 4.72 g.;  $\text{KH}_2\text{PO}_4$ , 2.72 g.; glucose, 2.0 g.; asparagine, 2.0 g.; and 1.7 mg. each of  $\text{FeCl}_2$ ,  $\text{MgCl}_2$ , and  $\text{CaCl}_2$ . It was adjusted to pH 7.0–7.1 with NaOH. The methionine-free mixtures of amino acids and growth factors contained all of the compounds tested in (1) except methionine.

The extent of growth was determined with a Photron reflectometer (6). The galvanometer deflections were calibrated in terms of total cell count with a Petroff-Hauser counting chamber. With our instrument one unit of galvanometer deflection equivalent to 13.4 million cells of *E. coli*/ml. The usual maximum growth is 900–1200 million cells/ml.

The organisms to be used in the studies of growth rates were incubated for 16 hours in "complete" medium at 25°C., centrifuged, washed, and resuspended in saline buffer at a concentration of 500 million cells/ml. The addition of 0.1 ml. of this suspension/10 ml. gave an initial concentration of 5 million cells/ml. Growth at 37°C. was followed turbidimetrically. In the presence of *l*-methionine a logarithmic rate of growth was obtained between 3 and 6 hours of incubation. The velocity constant (*K*) for the log. growth phase was calculated from the equation:

$$K = \frac{2.303}{T_2 - T_1} \log \frac{N_2}{N_1}$$

where *N* = millions of cells/ml.; *T* = hours of incubation. The compounds were added to the medium as sterile solutions for these series.

## RESULTS AND DISCUSSION

The response of the mutant to *l*-, *d*-, and *dl*-methionine<sup>2</sup> is shown in Fig. 1. The response to *l*-methionine was approximately linear and growth was essentially complete within 24 hours. The growth with *d*-methionine was slow. The turbidity at 24 hours was only about one-half of that shown at 64 hours. With excess *d*-methionine the response did not exceed one-third to one-half maximum within 3 days. In some experiments the growth increased slowly during longer incubation, but even after 2 weeks it did not equal that obtained with excess of the *l*-isomer. At concentrations below 2 γ/ml., *d*-methionine produced greater final growth than did *l*-methionine.

The acidity of the medium does not appear to be the factor limiting growth with excess *d*-methionine. During growth the reaction dropped

<sup>2</sup> The *l*-methionine was obtained from Eastman Kodak Co., and the *dl*-methionine from Merck and Co. The *d*-methionine was prepared by the method of du Vigneaud *et al.* (7) from a sample of *S*-benzyl-*d*-homocysteine, kindly provided by Dr. du Vigneaud.

to pH 4.6–4.8, the same value obtained with excess of the *l*-isomer. However, the turbidity was not increased by neutralization of these tubes after 24 hours of incubation or by the addition of 0.2 *M* phosphate buffer (pH 7) to the original medium to maintain the reaction above pH 6.5 during growth. The addition of a methionine-free mixture of amino acids and growth factors increased the turbidity obtainable with *d*-methionine 10–20%. The combination could be replaced by leucine, isoleucine, and threonine, each at a concentration of 50  $\gamma$ /ml.

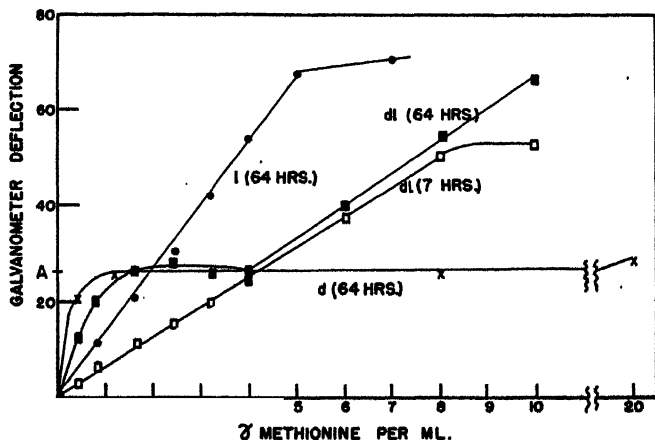


FIG. 1. The Response of Mutant 532-171 to Methionine Isomers in the Unsupplemented Basal Medium.

After 14 daily transfers in the presence of 10  $\gamma$  of *d*-methionine/ml. the maximum growth was about 60% of that with the *l*-isomer; however, this did not increase with further subculture.

The growth response to *dl*-methionine was a combination of the responses to the two components. After 7 hours of incubation, a straight-line response curve was obtained and *dl*-methionine was approximately one-half as active as *l*-methionine. At 24 hours the increased activity of the *d*-component was evident below about one-third maximal growth (level A, Fig. 1). After 64 hours the response below A approximated that obtained with *d*-methionine. Above this level the *dl*-methionine again showed one-half the activity of the *l*-isomer. It appears, therefore, that the mutant first rapidly utilizes the *l*-component of the *dl*-methionine. If the concentration of *dl*-methionine is

above 4  $\gamma$ /ml., the rapid growth with *l*-methionine raises the turbidity to level A before any significant amount of the *d*-isomer has been utilized. *dl*-Methionine is one-half as active as *l*-methionine under these conditions. With lower concentrations of *dl*-methionine, the amount of the natural isomer is not sufficient to raise the turbidity to level A, and the *d*-isomer is subsequently slowly utilized until level A is reached. These results with *dl*-methionine can be duplicated by mixtures of *d*- and *l*-methionine.

A definite explanation cannot be advanced for the greater final growth of the mutant with low concentrations of *d*-methionine than with the same quantity of the natural isomer. The rate of growth is much slower with *d*-methionine than with *l*-methionine. Thus, if *d*-methionine is converted to the natural isomer, this conversion must be the rate-limiting reaction and the amount of methionine used in unnecessary side reactions may be less under these conditions than when an equivalent quantity of *l*-methionine is added at the beginning of the growth period. We have observed that the response of the mutant to a hydrolyzate of cells grown with *d*-methionine is similar to that obtained with *l*-methionine. Also, this hydrolyzate acts like *l*-methionine in the presence of norleucine (see below). Thus, a major portion of the *d*-methionine was converted to some material with the growth properties of *l*-methionine.

#### *Activity of Methionine Derivatives*

A sample of the lithium salt of the  $\alpha$ -keto acid analogue of methionine,<sup>3</sup> which can replace methionine in the diet of the rat (8), showed an action on the mutant similar to that of *l*-methionine except that it had only 70–75% of the potency of *l*-methionine. Maximal growth was obtained, and no increased activity of the type shown by *d*-methionine was seen with the lower levels. Meyers and Porter (9) have reported that the keto acid did not serve as a sulfur source for *Proteus morganii* in continued subculture. Mutant 532-171 could be carried through 10 daily transfers on the basal medium containing 5  $\gamma$  of the keto acid/ml. No noticeable change in the rate of growth or quantitative requirement

<sup>3</sup> This sample was obtained through the generosity of Dr. H. Waelsch of the New York State Psychiatric Institute and Hospital. It was prepared by the action of amino-acid oxidase from kidney on *dl*-methionine and contained 80% keto acid on the basis of the hydrazone formed. Analysis of the preparation indicated less than 0.5% N, or a maximum *dl*-methionine content of 5.3%.

of the keto acid occurred. Thus the mutant appears to utilize the  $\alpha$ -keto analogue with approximately the same efficiency as *l*-methionine.

Turbidity measurements after 24 hours of incubation indicated *dl*-homocystine to be about one-tenth as active on a weight basis as *l*-methionine, and measurements at 72 hours indicated one-third of the activity. *dl*-Homocystine<sup>4</sup> supported slightly less final growth per unit amount than did homocystine. At a concentration of 100  $\gamma$ /ml. either compound supported maximal growth, and when present in low concentrations each gave disproportionately high final growth. The addition of choline (10–100  $\gamma$ /ml.) or of a mixture of pteroylglutamic acid (1  $\gamma$ /ml.), nicotinic acid (10  $\gamma$ /ml.), and thiamine, riboflavin, inositol, choline, *p*-aminobenzoic acid, calcium pantothenate, biotin, and pyridoxine (all in the concentrations listed in (1)) did not increase the response of the mutant to either of these substances.

Dehydromethionine<sup>5</sup> [ $\text{CH}_3\text{S}^+-\text{CH}_2\text{CH}_2\text{CH}(\text{COO}^-)-\text{NH}$ ] had 90–95% of the molar growth activity of *l*-methionine at low concentrations (1  $\gamma$ /ml.) but only 60% at the 5  $\gamma$ /ml. level even on the basis of turbidity at 3 days. The growth of the mutant in the presence of 2  $\gamma$  of *l*-methionine/ml. was prevented for 3 days by the addition of 1 mg./ml. of this compound. Methionine methylsulfonium bromide and iodide,<sup>5</sup> acetylmethionineallylsulfonium bromide, cystine, cysteine, cystathionine, glutathione, sodium thioglycollate, thiourea, and sodium sulfide were inactive at 100  $\gamma$ /ml. in the presence or absence of 2  $\gamma$  of *l*-methionine/ml. The addition of methyl mercaptan at concentrations of 0.5–1000  $\gamma$ /ml. did not affect the growth in the presence of 2  $\gamma$  of *l*-methionine/ml. and a methionine-free amino acid mixture. The sulfone derived from *dl*-methionine was unable to support growth of the mutant at concentrations of 0.1–100  $\gamma$ /ml. The response to 1  $\gamma$  of *l*-methionine/ml. was inhibited for 24 hours by 100  $\gamma$ /ml. of the sulfone but not by 10  $\gamma$ /ml. These compounds were sterilized by filtration and added to the basal medium aseptically.

Whereas the sulfone derived from methionine is inactive for the rat (11), the sulfoxide is able to replace methionine in its diet (12). A series of isomeric sulfoxides<sup>5</sup> were tested with the mutant. *L*- $\gamma$ -Methyl-

<sup>4</sup> This compound was generously furnished by Dr. E. Borek of the New York State Psychiatric Institute and Hospital.

<sup>5</sup> We wish to thank Dr. G. Tocannies and Dr. T. F. Lavine of the Lankenau Hospital, Philadelphia, for furnishing samples of the sulfoxides and sulfone derived from methionine, dehydromethionine (10), and the sulfonium compounds.



sulfinyl-*l*- $\alpha$ -aminobutyric acid (*ll*-sulfoxide) was of identical activity with *l*-methionine. Growth with *d*- $\gamma$ -methylsulfinyl-*l*- $\alpha$ -aminobutyric acid (*dl*-sulfoxide) was slow. However, concentrations below 2  $\gamma$ /ml. supported greater final growth than did *l*-methionine. Thus, the mutant appears to be able to reduce the *ll*-sulfoxide to methionine much more readily than the *dl*-sulfoxide. A racemate containing the *ll*-sulfoxide and the *dd*-sulfoxide also produced disproportionately high final turbidity at growth levels below half-maximum.

### *The Effect of Growth Rate on the Efficiency of Methionine Utilization*

In preliminary experiments on the determination of methionine by means of the mutant it was noted that addition of a mixture of amino acids and vitamins, or of a yeast extract which had been freed of methionine by treatment with peroxide, permitted a more rapid growth of the mutant coupled with a lowered final turbidity in the presence of limiting concentrations of *l*-methionine. This inverse relation between growth rate and efficiency of methionine utilization was similar to the results with several of the slowly utilized methionine derivatives. To check this relation further, we measured the growth rate and final turbidity of the mutant with the several derivatives and media. These data are assembled in Table I. Although the absolute values obtained varied from one experiment to another, the ratios between the values were reproducible. A standard of  $1.33 \times 10^{-5}$  *M* *l*-methionine was run with each experiment. An average value for *K* of 0.88 (range 0.84–0.95) was obtained in 6 experiments. All other data were calculated to this basis.

In the unsupplemented basal medium the growth rate of the mutant was approximately the same with *l*-methionine, the  $\alpha$ -keto analogue, the *ll*-sulfoxide, or dehydromethionine and did not vary with concentration. Dehydromethionine is known to react with cysteine (10) and might be expected to support a smaller final growth per unit amount than do the other compounds. The addition of the amino acid-vitamin mixture or of the yeast extract increased the rate of growth with *l*-methionine and lowered the final turbidity with limiting concentrations. The growth rates with  $0.67 \times 10^{-5}$  *M* *d*-methionine or  $1.33 \times 10^{-5}$  *M* *dl*-sulfoxide were low but the final turbidity was greater than with an equivalent concentration of *l*-methionine. A similar relation was observed with low concentrations of *dl*-homocysteine or *dl*-homocystine.

TABLE I  
Relation between the Velocity Constant (*K*) and Final Growth  
of Mutant 532-171 on Methionine Derivatives<sup>a</sup>

Compound	Concentration <i>M</i> × 10 <sup>-3</sup> /l.	<i>K</i> <sup>b</sup>	Turbidity reading at 48 hrs.
<i>l</i> -Methionine	0.67 <sup>c</sup>	0.89	11
	1.33	0.88	24
	6.7	0.84	76
	1.33 <sup>d</sup>	1.18	19
	6.7 <sup>d</sup>	1.15	73
	1.33 <sup>e</sup>	0.99	19
	6.7 <sup>e</sup>	0.97	82
$\alpha$ -Keto analogue	1.33/	0.86	23
	6.7/	0.84	76
<i>d</i> -Methionine	0.67	0.29	20
	1.33	0.34	21
	6.7	0.36	26
<i>l</i> -Sulfoxide	1.33	0.87	23
<i>d</i> -Sulfoxide	1.33	0.42 (4-7 hrs.)	31
		0.58 (7-9 hrs.)	
Dehydromethionine	1.33	0.88	20
	6.7	0.86	74
<i>d</i> -Homocystine	0.33	0.10 (8-13 hrs.)	16
	1.67	0.27	34
	3.3	0.25	40
	10.0	0.57	73
	20.0	0.67	81
<i>d</i> -Homocysteine	0.67	0.12 (6-10 hrs.)	13
	1.33	0.15 (6-10 hrs.)	15
	6.7	0.39	36
	26.7	0.92	84
	80.0	0.49	81

<sup>a</sup> All tests run in triplicate.

<sup>b</sup> The 3-6 hr. values were used unless otherwise noted.

<sup>c</sup> One  $\gamma$  of *l*-methionine/ml.

<sup>d</sup> A methionine-free mixture of amino acids and vitamins was added to the basal medium in these tubes.

<sup>e</sup> Peroxide-treated yeast extract (2 mg./ml.) was added to these tubes.

<sup>f</sup> Calculated on the basis of 75%  $\alpha$ -keto analogue in the sample.

It appears, therefore, that conditions which increase the growth rate of the mutant decrease its final response to limiting concentrations of *l*-methionine and that, in a number of instances, compounds which support only slow growth are utilized more efficiently than is *l*-methionine.

The velocity constants obtained in the presence of *dl*-homocystine or *dl*-homocystine were dependent on the concentration of these substances. In the presence of high levels of these compounds the rates approximated those observed with *l*-methionine. This dependence of the growth rate on the concentration of homocystine indicates that this substance is probably active for the mutant only after conversion to methionine, and this conversion is the reaction limiting the rate of growth in the presence of homocystine.

#### *Application of Mutant 532-171 to Microbiological Assay*

It seemed possible to determine methionine by means of mutant 532-171 if *l*-methionine were used as a standard. The usual bacteriological procedures were employed. When peptone or yeast extract was assayed in the unsupplemented basal medium a pronounced increase in the apparent methionine content was observed with increasing sample levels. The addition of a methionine-free amino acid mixture reduced this shift but did not eliminate it. We therefore attempted to remove the methionine from natural materials. A "methionine-free" casein hydrolyzate was prepared by the method of Albanese (13) but variable high blanks and high methionine recoveries were obtained in its presence. This may be due to sulfoxide remaining after the hydrogen peroxide-sulfuric acid treatment (14). The addition of molybdate has been shown to catalyze the further oxidation of the sulfoxide to the sulfone (15) which cannot be utilized by our organism.

Methionine-free casein hydrolyzate, peptone, and yeast extract were prepared by the following procedure using molybdate. To 20 g. of the material dissolved in 80 ml. of water were added 4 ml. of 10 *N* sulfuric acid, 4 ml. of 0.5 *M* ammonium molybdate, and 1 ml. of 30% hydrogen peroxide (Superoxol), and the volume adjusted to 100 ml. Four ml. of peroxide were required to obtain a methionine-free product from the yeast extract. After 18 hours at 30°C. the solutions were adjusted to pH 7 with ammonia and stirred with 1 g. of manganese dioxide for 10 minutes. The solutions were filtered and diluted to 200 ml. Thus, 1 ml. was equivalent to 100 mg. of the original material. At a concentration of 10 mg./ml. of the final medium these preparations did not support growth of the mutant.

The effect of these supplements on the apparent methionine content of bacto-peptone and casein is given in Table II. On the basal medium (A) recoveries were erratic and a pronounced drift in the apparent methionine content occurred with the peptone. The addition of the peroxide-treated Bacto-peptone (B) or casein hydrolyzate (C) did not improve the results. The peroxide-treated yeast extract (D) eliminated the drift and permitted excellent recoveries. The methionine value we have obtained for casein (3.09%) is of the same magnitude as other reported analyses (*cf.* (16)). The addition of all three preparations (E)

TABLE II  
*Effect of Supplementation of the Basal Medium on Apparent  
Methionine Content of Peptone and Casein*

Medium <sup>a</sup>	Bacto-peptone <sup>b</sup>		Casein, Hammarsten <sup>c</sup>	
	Methionine found	Added methionine recovered	Methionine found	Added methionine recovered
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
A	0.393	114, 114	3.32	96, 132
B	0.449	72, 75	3.30	89, 89
C	0.356	75, 82	3.24	95, 101
D	0.321	97, 103	3.09	98, 104
E	0.328	100, 104	3.09	94, 100

<sup>a</sup> Medium A: Basal medium.

Medium B: Basal medium + 2 mg./ml. H<sub>2</sub>O<sub>2</sub>-treated Bacto-peptone.

Medium C: Basal medium + 2 mg./ml. H<sub>2</sub>O<sub>2</sub>-treated Casein hydrolyzate.

Medium D: Basal medium + 2 mg./ml. H<sub>2</sub>O<sub>2</sub>-treated Yeast extract.

Medium E: Basal medium + 2 mg./ml. of each of supplements in B, C, and D.

<sup>b</sup> Unhydrolyzed sample.

<sup>c</sup> One hundred mg. of the sample were autoclaved for 10 hrs. in a sealed tube with 5 ml. of 10% HCl and neutralized with 5 N NaOH. The calculated methionine content is for the ash- and water-free sample.

gave no further improvement. Therefore, in subsequent experiments the yeast extract alone was used at a concentration of 2 mg./ml. In separate tests the growth of mutant 532-171 in the basal medium with 2  $\gamma$  of *l*-methionine/ml. was not inhibited by  $3 \times 10^{-3}$  M ammonium molybdate. Since the final molybdate concentration is only  $2 \times 10^{-4}$  M, no attempt was made to remove the molybdate from the preparations. If complete conversion to the sulfone is assumed for the 0.75% methionine initially present in the yeast extract sample (as assayed with

the mutant), the final concentration of the sulfone in the supplemented medium is 18  $\gamma$ /ml. This concentration did not affect the response to *l*-methionine in tests with the basal medium.

Fig. 2 illustrates the effect of the peroxide-treated yeast extract on the response of the mutant to *l*-methionine (curves A and C). The turbidity obtained with limiting concentrations of *l*-methionine was reduced about one-third by the yeast extract. When a methionine-free mixture of amino acids and vitamins was used instead, the reduction

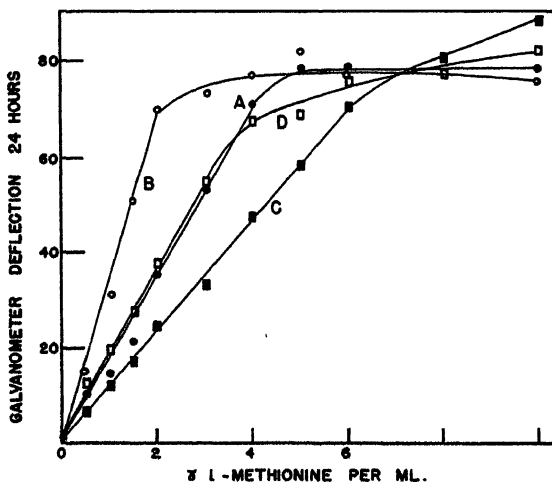


FIG. 2. The Effect of Peroxide-Treated Yeast Extract and Norleucine on the Response of Mutant 532-171 to *l*-Methionine.

A, Basal medium;

B, Basal medium with 50  $\gamma$  *dl*-norleucine/ml.;

C, Basal medium with 2 mg. peroxide-treated yeast extract/ml.;

D, Basal medium with peroxide-treated yeast extract and *dl*-norleucine.

in turbidity was about one-fifth. The assay values in the yeast extract basal medium showed a maximum variation of about 10% at varying levels of the test material (Table III).

In subsequent work it was noted that the addition of 50  $\gamma$  of *dl*-norleucine/ml. increased the response of the mutant to suboptimal levels of *l*-methionine, both in the presence and absence of the peroxide-treated yeast extract (Fig. 2). The activity of *d*-methionine, homocystine, and the *dl*-sulfoxide was sharply reduced (17). In the yeast extract medium containing 50  $\gamma$  of norleucine/ml., *dl*-methionine has

TABLE III

*Methionine Content of Materials Calculated from Different Assay Levels<sup>a</sup>*

Bactopeptone			Hydrolyzed bactopeptone <sup>b</sup>			Yeast extract			Hydrolyzed yeast extract <sup>b</sup>		
Sample mg.	Methionine		Sample mg.	Methionine		Sample mg.	Methionine		Sample mg.	Methionine	
	$\gamma$	per cent		$\gamma$	per cent		$\gamma$	per cent		$\gamma$	per cent
5.0	17.4	0.35	2.5	22.9	0.92	2.0	15.2	0.76	2.0	16.4	0.82
7.5	26.1	0.35	5.0	51.5	1.03	3.0	21.1	0.70	3.0	22.5	0.75
10.0	38.7	0.39	7.5	74.0	0.99	4.0	31.0	0.78	4.0	29.5	0.74
Average		0.36			0.98			0.75			0.77

<sup>a</sup> All tubes contain 2 mg. of peroxide-treated yeast extract/ml. Ten ml. volumes in all tubes.

<sup>b</sup> 100 mg. of the sample were autoclaved 10 hrs. in a sealed tube with 5 ml. of 10% HCl and neutralized with 5 N NaOH.

shown 50–52% of the activity of the *l*-isomer in three experiments. The mechanism of the action of norleucine is discussed in Paper II of this series (17).

It seemed likely that the specificity of the methionine assay would be improved if norleucine were present in the basal medium. However, the addition of 50  $\gamma$  of norleucine/ml. actually increased the apparent methionine content of the Bactopeptone from 0.32% to 0.39% and that of the yeast extract from 0.74% to 0.81%. The peroxide-treated yeast extract was present in all tubes. It will be necessary to test proteins of known methionine content to decide which procedure yields the correct values.

The effect of norleucine on the activity of the isomers of methionine does not appear to have been considered in previous studies of the assay of methionine. Shankman *et al.* (18) with *L. arabinosus* and Greenhut *et al.* (19) using *Strep. faecalis* apparently obtained normal standard curves with *dl*-methionine in the absence of norleucine. In media containing norleucine, *dl*-methionine was half as active as the *l*-isomer for *Strep. faecalis* (20), *L. arabinosus* (16), or *Leuc. mesenteroides* (16) but had the same activity as *l*-methionine for *L. fermenti* (16). In none of these experiments were the isomers of methionine tested in the presence and absence of norleucine.

A similar effect of supplements in reducing the efficiency of utilization of an essential substance while increasing the rate of growth has not, to our knowledge, been previously reported. The usual procedure in preparing assay media is to add as complete a group of supplements as possible, hoping thereby to improve the sensitivity of the assay. Our data show that the reverse may actually occur. If the organism is to be used for assay, the addition of supplements is still necessary, however, to prevent, if possible, obtaining such an effect from the test sample. The specificity of the assay may also be altered by such supplementation.

### SUMMARY

The response of a methionine-requiring mutant (532-171) of *E. coli* to *l*-, *d*-, and *dl*-methionine and to various related compounds is described. The mutant does not attain maximal growth in the presence of excess *d*-methionine, however, the final growth with low concentrations of this isomer being greater than with the same concentration of *l*-methionine. There appears to be an inverse relation between the rate of growth of this organism and the efficiency of its utilization of methionine.

The use of the mutant for the determination of methionine has been studied. The preparation of methionine-free crude materials and their effect on the response to methionine is described.

*dl*-Norleucine increases the final growth attained by the mutant in the presence of limiting concentrations of *l*-methionine.

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## Studies on the Sulfur Metabolism of *Escherichia coli*.

### II. Interrelations of Norleucine and Methionine in the Nutrition of *Escherichia coli* and of a Methionine-Requiring Mutant of *Escherichia coli*

J. O. Lampen and M. J. Jones

*From the Chemotherapy Division, Stamford Research Laboratories,  
American Cyanamid Company, Stamford, Connecticut*

Received December 18, 1946

#### INTRODUCTION

Norleucine ( $\alpha$ -aminocaproic acid) has been reported by Harris and Kohn (1) as preventing the growth of a strain of *Escherichia coli* by competitively inhibiting its utilization of methionine. The inhibition of the growth of *Proteus morganii* by norleucine is specifically antagonized by methionine and certain of its derivatives (2). Our strain of *Escherichia coli*<sup>1</sup> had also been observed to be extremely sensitive to norleucine. During studies on the utilization of methionine by a mutant strain (532-171)<sup>1</sup> of *Escherichia coli* which has lost the ability to synthesize this compound, we observed that the final turbidity attained by this mutant in the presence of suboptimal amounts of *l*-methionine was unexpectedly increased by the addition of 50  $\gamma$  of *dl*-norleucine/ml. (3).

Our previous experiments with *Escherichia coli* had been performed with media in which the organism was forced to synthesize its own requirements of methionine. The mutant strain, however, had been utilizing the preformed molecule for its growth. It seemed possible that the point of inhibition of our strain of *Escherichia coli* by norleucine was the synthesis of methionine rather than its utilization.

The experiments reported in this paper were designed to determine the point of action of the norleucine and to clarify the mechanism of the

<sup>1</sup> Our stock strain of *E. coli* (No. 15) is No. 9723 of the American Type Culture Collection and mutant 532-171 (derived from *E. coli* (No. 15)) is No. 9663.

increased response to methionine observed with the mutant strain in the presence of norleucine.

## EXPERIMENTAL AND RESULTS

### *Cultures and Techniques*

The organisms used in these experiments and the bacteriological procedures and media have been described in previous papers (3, 4). The methionine derivatives were those used in Paper I of this series (3). Inocula of about 50,000 cells/ml. were employed in all experiments except those in which the rate of growth was determined. Five million cells/ml. were added for the latter tests. Unless otherwise stated, all supplements were added to the basal medium before autoclaving. With the turbidimeter used one unit of galvanometer deflection is equal to  $1.34 \times 10^7$  cells/ml.

### *The Action of Norleucine on Escherichia coli*

The concentration of norleucine required to produce half-maximal inhibition<sup>2</sup> of the growth of *Escherichia coli* in the unsupplemented basal medium was 1–1.5  $\gamma$ /ml. at 16 hours and 3–4  $\gamma$ /ml. after 72 hours of incubation. A reasonably constant norleucine:methionine inhibition ratio was obtained over a 64-fold range of norleucine concentration after 16 or 42 hours of incubation (Table I). In several experiments, using 32–512  $\gamma$  of norleucine/ml., we have obtained inhibition ratios of 35:1 to 100:1 at 16 hours and 125:1 to 250:1 after two and three days. The shift in the inhibition ratio with time observed with the lowest level of norleucine in this and similar experiments may be the result of a slow synthesis of methionine by *Escherichia coli*. This synthesis is probably not significant in the presence of higher concentrations of norleucine.

The amount of *l*-methionine required for the antagonism of 8  $\gamma$  of norleucine/ml. is much less than that required for half-maximal growth by the mutant under these conditions (*cf.* Table III). Thus the growth of *E. coli*, in this instance at least, is probably still dependent on its synthesis of methionine. If norleucine prevented only the synthesis of methionine, all levels should be antagonized by a constant concentration of methionine, *i.e.*, the concentration of the preformed molecule required for the growth of the organism. Instead, a nearly constant inhibition ratio was obtained. It appears, therefore, that the primary action of norleucine with the parent *Escherichia coli* must be against the utilization of methionine rather than its synthesis.

<sup>2</sup> Half-maximal inhibition of growth indicates the reduction of the turbidity response at the given time to one half of that obtained in the control tube.

TABLE I

*The Antagonism by Methionine of Norleucine Inhibition of Escherichia coli*

Hours of incubation	$\gamma$ <i>dl</i> -Norleucine/ml.			
	8	32	128	512
	$\gamma$ /ml. of <i>l</i> -methionine yielding half-maximal antagonism			
16	0.1	0.8	1.5	6
42	0.02	0.2	1.0	3
68	0.003	0.2	0.8	3
	Inhibition ratios <sup>a</sup>			
16	65	38	84	85
42	250	145	125	170
68	1670	145	156	170

<sup>a</sup> The inhibition ratio represents the  $\gamma$  of norleucine antagonized by 1  $\gamma$  of methionine. All norleucine concentrations are corrected for the 1.5  $\gamma$ /ml. required for half-maximal inhibition of growth on the basal medium at 16 hours and the 3.0  $\gamma$ /ml. required at 42 and 68 hours.

*The Action of Norleucine on Mutant 532-171*

The final turbidity attained by mutant 532-171 in the presence of suboptimal concentrations of *l*-methionine was increased 60–100% by the addition of 50  $\gamma$  of norleucine/ml. (*cf.* Fig. 3 of (3)). In similar experiments, using a medium supplemented with a peroxide-treated yeast extract (3), increases of 30–60% were observed. The same results were obtained when the norleucine and methionine were added as sterile solutions as when they were autoclaved together in the medium. Norleucine did not support growth of the mutant in the absence of *l*-methionine.

Table II illustrates the effect of norleucine on the response of the mutant to methionine derivatives. The effect of norleucine on the final growth with  $\alpha$ -keto analogue, the *ll*-sulfoxide, or the *ld*-sulfoxide, was similar to its action in the presence of *l*-methionine, although the increase was observed with the *ld*-sulfoxide only after 112 hours. Growth with *d*-methionine or homocystine was completely inhibited for 112 hours. These data do not necessarily indicate a specific action

TABLE II

*The Effect of Norleucine on the Growth of Mutant 532-171 in the Presence of Compounds Related to L-Methionine*

Compound	(γ/ml.)	Norleucine γ/ml.	Turbidity		
			16 hrs.	48 hrs.	112 hrs.
<i>L</i> -Methionine	2	—	22 <sup>a</sup>	31	—
	2	50	36	55	—
$\alpha$ -Keto analogue <sup>b</sup>	2	—	19	25	—
	2	50	42	49	—
<i>d</i> -Methionine	10	—	12	38	48
	10	50	0	0	0
<i>ll</i> -sulfoxide <sup>c</sup>	2	—	19	27	—
	2	50	34	53	—
<i>dl</i> -sulfoxide <sup>d</sup>	2	—	23	30	32
	2	50	1	30	48
Homocystine	10	—	9	26	38
	10	50	1	1	2

<sup>a</sup> All values are averages of duplicate tubes.

<sup>b</sup> Added as a sterile solution.

<sup>c</sup> *l*-γ-Methylsulfinyl-*l*-α-aminobutyric acid.

<sup>d</sup> *d*-γ-Methylsulfinyl-*l*-α-aminobutyric acid.

of the norleucine against the conversion of *d*-methionine or homocystine to *L*-methionine which presumably occurs during the utilization of these substances. This conversion is slow even in the absence of norleucine and the *L*-methionine concentration is probably very low at all times. Thus a greater inhibition of growth would be expected here than occurs in the presence of an equivalent quantity of *L*-methionine.

An experiment was performed to determine the effect of varying concentrations of norleucine on the response to *L*-methionine (Table III). The turbidity was increased markedly by norleucine concentrations above 0.3 γ/ml. High levels of norleucine, however, inhibited the growth of the mutant. A norleucine:methionine ratio of 300:1 was necessary to prevent visible growth for 17 hours. In another test with 10 γ of *L*-methionine/ml., which supports maximal growth of the

mutant, the turbidity at 16 hours was unaltered in the presence of 1,000  $\gamma$  of norleucine/ml. Thus, high concentrations of norleucine can exert an inhibitory action which appears to be directed against the utilization of methionine, since the latter compound can antagonize the inhibition.

TABLE III

*The Effect of Varying Amounts of Norleucine on the Response of Mutant 532-171 to l-Methionine*

Norleucine $\gamma$ /ml.	l-Methionine			
	1 $\gamma$ /ml.		3 $\gamma$ /ml.	
	17 hrs.	40 hrs.	17 hrs.	40 hrs.
1,000	0*	45	0	77
300	0	35	82	78
100	27	36	94	93
30	39	43	88	89
10	29	30	75	86
3	28	31	66	69
1	22	24	63	65
0.3	17	19	57	65
0.1	16	19	59	60
0	15	18	56	60

\* Turbidity readings.

Table IV illustrates the effect of the addition of 50  $\gamma$  of norleucine/ml. on the log phase growth rate (3) of the mutant in the presence of *l*-methionine or its sulfoxides. When a suboptimal level of *l*-methionine (2  $\gamma$ /ml.) was used, the addition of norleucine reduced the growth rate 10–15% on either the basal or supplemented medium. The rate obtained in the presence of the *ll*-sulfoxide was reduced 36% by norleucine and that with the *ld*-sulfoxide 95%. The final turbidity attained in each of these instances was increased 50–100% by the addition of the norleucine. The growth rate with excess *l*-methionine was not affected by the addition of 50  $\gamma$  of norleucine/ml.

It can be seen that a norleucine:*l*-methionine ratio of 300:1 was required to prevent the growth of the mutant for 17 hours (Table III), but that the growth rate was still suboptimal with a ratio of 25:1 (Table IV). In the presence of *l*-methionine concentrations of 0.5–5.0  $\gamma$ /ml., as customarily used in a standard response curve, the

TABLE IV

*The Effect of Norleucine on the Growth Rate of Mutant 532-171 in the Presence of L-Methionine or its Sulfoxides*

Compound <sup>a</sup>	(γ/ml.)	Velocity constant (K)			
		Basal medium		Basal medium and peroxide-treated yeast extract <sup>b</sup>	
		Control	+NL <sup>c</sup>	Control	+NL <sup>c</sup>
<i>L</i> -Methionine	2	0.88	0.80	0.99	0.84
	2	0.88	0.75	—	—
	10	0.84	0.84	0.97	1.01
	10	0.88	0.85	—	—
<i>ll</i> -Sulfoxide	2	0.87	0.56	—	—
<i>dl</i> -Sulfoxide	2	0.58	0.03	—	—

<sup>a</sup> Sterilized by filtration and added to the medium aseptically.

<sup>b</sup> Two mg. of peroxide-treated yeast extract (3)/ml.

<sup>c</sup> NL indicates *dl*-norleucine, 50 γ/ml.

addition of 50 γ of norleucine/ml. yields ratios of 100:1 to 10:1. Thus the conditions required for an increased final response of the mutant to methionine were those under which there was a partial inhibition of the rate of growth. This is in accord with the inverse relation between the rate of growth and the efficiency of methionine utilization noted in previous experiments with this mutant (3).

At least two explanations appear possible for the increased final response to methionine obtained with the mutant under these conditions of slower growth. Norleucine may replace methionine in some of its less essential reactions, sparing a larger net amount of methionine for necessary syntheses. Or, under these conditions of partial inhibition, those reactions of methionine less essential to the cell may be inhibited to a greater extent than the more essential processes. Such reactions might include the utilization of the carbon chain or of the amino group or the formation of cystine from the sulfur of methionine. The use of methionine for these purposes may be considered wasteful since other materials can be used as sources of energy or of carbon and nitrogen, and cystine can ordinarily be synthesized by *E. coli* through other reactions (5). Thus, the final products may be essential, but their formation from methionine is not. The result of this prevention of unnecessary side reactions would be to increase the amount of cellular material ultimately synthesized per unit of methionine.

A partial reduction of the rate of growth is not the only requirement for an increased efficiency of methionine utilization by the mutant. Methoxinine (6) and norvaline (1, 7) both appear to inhibit the utilization of methionine by the mutant and by *E. coli*, but at partially inhibitory concentrations methoxinine increased the final response to *l*-methionine whereas norvaline did not (7). It was noted that a methionine-free mixture of amino acids (3) antagonized the action of norvaline on *E. coli* much more readily than that of norleucine (7). Some difference in specificity, such as that just mentioned, may be the reason for the difference between the effects of these compounds on the efficiency of the utilization of methionine by the mutant.

### SUMMARY

The inhibition of *Escherichia coli* by *dl*-norleucine is reversed by *l*-methionine. A reasonably constant norleucine:methionine inhibition ratio was obtained over a considerable range of norleucine concentration. The amounts of *l*-methionine required for the antagonism were much less than those required by mutant 532-171 for half-maximal growth under similar conditions. It appears, therefore, that norleucine inhibits primarily the utilization, rather than the synthesis, of methionine by *E. coli*.

The final turbidity attained by mutant 532-171 in the presence of suboptimal levels of *l*-methionine is increased by concentrations of norleucine which produce a partial reduction of the rate of growth. Norleucine prevents growth for 16 hours when the norleucine:methionine ratio reaches 300:1. The mechanism of the increased efficiency of methionine utilization by the mutant in the presence of partially inhibitory concentrations of norleucine is discussed.

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## Studies on the Sulfur Metabolism of *Escherichia coli*.

### III. Mutant Strains of *Escherichia coli* Unable to Utilize Sulfate for their Complete Sulfur Requirements

J. O. Lampen,<sup>1</sup> R. R. Roepke and M. J. Jones

*From the Chemotherapy Division, Stamford Research Laboratories,  
American Cyanamid Company, Stamford, Connecticut*

Received December 18, 1946

#### INTRODUCTION

*Escherichia coli* is normally able to synthesize its entire requirements of sulfur-containing substances from inorganic sulfur of any oxidation level. We have obtained a series of mutant strains of *E. coli* No. 15 which have lost the ability to grow with sulfate as their only source of sulfur. Certain of these mutants require the addition of methionine for growth, whereas others can utilize either cystine or methionine. Several strains are able to utilize a variety of sulfur-containing materials; e.g.,  $\text{Na}_2\text{S}$ ,  $\text{Na}_2\text{S}_2\text{O}_3$ ,  $\text{Na}_2\text{S}_2\text{O}_4$ , cystine, etc. Such organisms, which require a more reduced form of sulfur than that of sulfate, are termed parathiotrophic (1) in contrast to the normally euthiotrophic *E. coli*.

Similar parathiotrophic mutants were obtained by Fries (2) after X-irradiation of the fungus *Ophiostoma multiannulatum*. One strain was able to use sulfite while four strains were unable to do so. Parathiotrophy has also been observed in the *Saprolegniaceae* (3) and in *Staphylococcus aureus* (4). An analogous mutant strain of *Neurospora* unable to reduce nitrate to nitrite has been described (5).

This report deals with an attempt to elucidate some of the steps involved in the sulfur metabolism of *E. coli* from a study of mutant strains which require a source of sulfur other than sulfate.

<sup>1</sup> Present address: Department of Biological Chemistry, Washington University, St. Louis, Missouri.

## EXPERIMENTAL AND RESULTS

*Organisms and Techniques*

The media, materials, and experimental techniques are described in the accompanying paper (6). The sulfates of the usual basal medium were replaced by equivalent amounts of the respective chlorides for the low-sulfur basal medium. Unless otherwise stated, all supplements were sterilized by filtration and added to the medium aseptically.

Mutants 532-171, 754M-171, 1251-171, 654-228, 932-230, and 1590-230 have been previously reported (7). The metabolism of mutant 532-171 is described in more detail in papers I and II of this series (6, 8). Mutant 1-344<sup>2</sup> was obtained by serial passage of *E. coli* in the medium of Kohn and Harris (9) in the presence of increasing concentrations of sulfanilamide. Mutant 1-273-384 resulted from a similar serial passage of mutant 273-384, which requires preformed *p*-aminobenzoic acid (10). For this series 0.002  $\gamma$  of *p*-aminobenzoic acid was added/ml. of medium. The other mutants were isolated from X-rayed resting cell suspensions (in saline-phosphate buffer).

*Response of the Mutant Strains to Sulfur-Containing Compounds*

Table I summarizes the growth of the mutant strains and of the parent *E. coli* in the presence of various sulfur-containing compounds. Each of the tests has been performed at least twice. The parent *E. coli* reached only about 10% of maximal growth on the low-sulfur basal medium. It was able to utilize all of the substances tested. Three mutants grew with a variety of inorganic compounds containing sulfur of a lower oxidation state than that of sulfate. One of these (1590-230) could not utilize added sulfite. Maximal growth of these mutants or of the parent *E. coli* did not occur within a three-day period with *dl*-methionine even when this substance was present in a concentration of 1,000  $\gamma$ /ml. *d*-Methionine possessed only slight activity.

Two mutants (508-462 and 255-468) were unable to utilize the inorganic sulfur compounds or thioglycollate but grew well in the presence of cysteine or cystine. These strains were able to grow in the presence of cystathionine, homocysteine, or methionine, but this growth was slower than in the presence of cystine. Mutant 508-462 did not grow in the presence of a methionine- and cystine-free mixture of amino acids and growth factors (as listed in (7)) with the addition of 1 mg. of pyruvic acid and 0.1 mg. of Na<sub>2</sub>S per ml. Mutant 255-468 grew slowly, but on subculture from this medium growth occurred in the unsupplemented basal medium.

<sup>2</sup> Roepke, R. R., To be published.

TABLE I  
*The Growth of Mutant Strains of E. coli with Various  
 Sulfur-Containing Materials*

Cultures <sup>a</sup>	Supplement (100 $\gamma$ /ml.)							
	Na <sub>2</sub> SO <sub>4</sub>	Na <sub>2</sub> SO <sub>3</sub>	Na <sub>2</sub> S <sub>2</sub> O <sub>4</sub> , Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> , or Na <sub>2</sub> S	Sodium thiogly- collate	<i>l</i> -Cystine or <i>l</i> -Cysteine	<i>l</i> -Cysta- thionine	<i>dl</i> -Homo- cystine or <i>dl</i> -Homo- cysteine <sup>b</sup>	<i>dl</i> -Meth- ionine
<i>E. coli</i>	4 + <sup>c</sup>	4 +	4 +	4 +	4 +	4 +	2 +	2 +
1251-171	—	4 +	4 +	2 +	4 +	4 +	2 +	2 +
932-230	—	4 +	4 +	2 +	4 +	2 +	2 +	±
1590-230	—	—	4 +	2 +	4 +	1 +	2 +	±
508-462	—	—	—	—	4 +	4 +	4 +	1 +
255-468	—	—	—	— <sup>d</sup>	4 +	2 +	1 +	1 +
532-171	—	—	—	—	—	—	4 +	4 +
754M-171	—	—	—	—	—	—	4 +	4 +
282-460	—	—	—	—	—	—	4 +	4 +
495-460	—	—	—	—	—	—	4 +	4 +
654-228	—	—	—	—	—	—	±	4 +
3-301	—	—	—	—	—	—	±	4 +
1-344	—	—	—	—	—	—	—	4 +
1-273-384 <sup>e</sup>	—	—	—	—	—	—	—	4 +

<sup>a</sup> The low-sulfur basal medium was used for *E. coli*; all other strains were tested in the regular basal medium.

<sup>b</sup> The addition of 20  $\gamma$  of choline chloride/ml. did not affect these results.

<sup>c</sup> Turbidity after 44-48 hrs. was graded as —, ± (doubtful growth), and 1 + to 4 +.

<sup>d</sup> Adaptive; see Discussion.

<sup>e</sup> PABA (0.1  $\gamma$ /ml.) added to all tubes.

All of the remaining mutants grew well in the presence of methionine. The data of Table II show that the concentration of *l*-methionine required for growth was the same with all strains. The  $\alpha$ -keto analogue of methionine had activity equivalent to that of *l*-methionine. Four of the mutants attained maximal growth with homocystine although the rates of growth varied rather widely. The others gave at best light growth which could not be subcultured (Table II). *dl*-Homocystine proved to be slightly less active than *dl*-homocystine. Choline was added in the experiments with homocystine and homocysteine to insure optimal conditions. However, the response of mutant 532-171 to limiting concentrations of these substances was not increased by the addition of choline (6).

Methyl mercaptan was tested as a precursor of methionine for these mutants because of the report by Waelsch and Borek (11) that the  $\alpha$ -keto analogue of methionine yields this mercaptan on hydrolysis. Concentrations of 0.5–1,000  $\gamma$ /ml. did not support the growth of mutants 532-171 or 1-344 nor did they affect the growth of these strains in the presence of 2  $\gamma$  of *l*-methionine/ml. The addition of 10  $\gamma$  of methyl mercaptan/ml. prevented the growth of *E. coli* and of mutants 932-230 and 255-468 for 24–48 hours. In the experiments with the latter two mutants, cystine was present at a concentration of 10  $\gamma$ /ml. The action of the mercaptan on *E. coli* was antagonized by *l*-cystine, *dl*-methionine, or *dl*-homocystine.<sup>3</sup>

TABLE II

*The Response of Various Methionine-Requiring Mutants to l-Methionine and Related Compounds*

The unsupplemented basal medium was used.

Mutant	<i>l</i> -Methionine		$\alpha$ -Keto analogue <sup>a</sup>		<i>dl</i> -Homocystine + choline ( $1 \times 10^{-4}$ M)		
	$1 \times 10^{-5}$ M	$5 \times 10^{-5}$ M	$1 \times 10^{-5}$ M	$5 \times 10^{-5}$ M	$2 \times 10^{-5}$ M	$1 \times 10^{-4}$ M	$4 \times 10^{-4}$ M
532-171	23 <sup>b</sup>	68	21	63	17	43	62
754M-171	20	61	17	74	24	47	56
282-460	22	69	21	73	0	38	63
495-460	23	58	23	65	9	37	57
654-228	27	61	26	66	0	4 <sup>c</sup>	6 <sup>c</sup>
3-301	19	48	16	69	0	0	4 <sup>c</sup>
1-344	16	53	16	58	0	0	0
1-273-384 <sup>d</sup>	19	42	22	46	0	0	0

<sup>a</sup> All concentrations are based on the pure compound. The sample was assumed to contain 75% of the  $\alpha$ -keto analogue (cf. (6)).

<sup>b</sup> Turbidity readings at 24 hrs. One unit is equivalent to 13.4 million cells/ml.

<sup>c</sup> No visible growth on subculture under the same conditions.

<sup>d</sup> PABA (0.1  $\gamma$ /ml.) added to all tubes.

### *Experiments with Combinations of Cystine and Methionine*

The addition of *dl*-methionine increased the response of mutants 932-230 and 1590-230 to limiting concentrations of cystine (Fig. 1). Mutant 932-230 required about 5.3  $\gamma$  of cystine sulfur/ml. for growth on the basal medium (Curve A) but only 1–1.5  $\gamma$ /ml. in the presence

<sup>3</sup> Lampen, J. O., and Jones, M. J., Unpublished data.

of 10  $\gamma$  of *dl*-methionine/ml. (Curve D). Curve B shows that certain combinations of cystine and methionine sulfur supported greater growth than did an equal amount of cystine sulfur. This is demonstrated further in Fig. 2. In the presence of 1.07  $\gamma$  of cystine sulfur/ml. the sulfur of methionine was used more efficiently than was that of additional cystine. Similar results were obtained with mutant 1590-230.

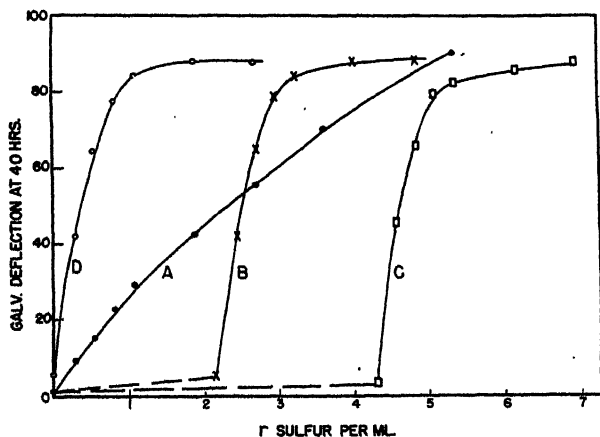


FIG. 1. The Effect of Methionine on the Response of Mutant 932-230 to Cystine.

All supplements were added to the basal medium before autoclaving. Solid line: cystine; broken line: methionine. A. Increasing concentrations of *l*-cystine. B. Ten  $\gamma$  of *dl*-methionine (2.15  $\gamma$  of sulfur)/ml. plus increasing concentrations of *l*-cystine. C. Twenty  $\gamma$  of *dl*-methionine (4.30  $\gamma$  of sulfur)/ml. plus increasing concentrations of *l*-cystine. D. As B except sulfur of *dl*-methionine not included in graph.

Good growth of the original isolate of mutant 255-468 occurred within 16 hours if both cystine and methionine were present but required 24 hours with cystine alone. The response to limiting concentrations of cystine was also increased in the presence of methionine. The response curves shown in Fig. 3 illustrate this. It is of interest that these strains and also the 3 mutants able to utilize sulfide are apparently able to synthesize methionine from added cystine more readily than cystine from added methionine. Mutants 508-462 and 255-468 were able to grow on the low-sulfur medium in the presence of cystine. The response to limiting concentrations of cystine on this medium was not affected by the addition of 10  $\gamma$  of  $\text{Na}_2\text{S}$ /ml. Thus,

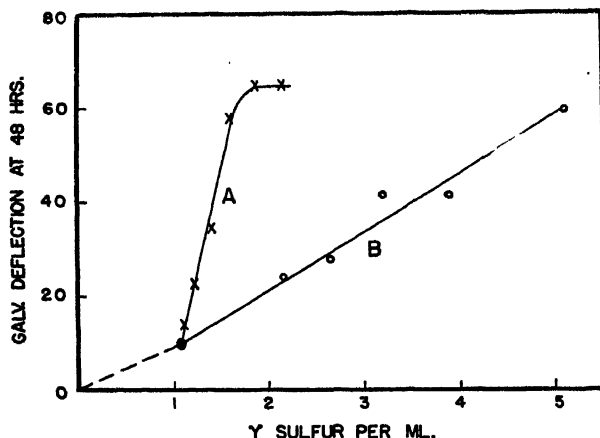


FIG. 2. The Response of Mutant 932-230 to Combinations of Cystine and Methionine.

The tubes of curves A and B contained 1.07  $\gamma$  of cystine sulfur (4.0  $\gamma$  of *l*-cystine)/ml. with graded amounts of *dl*-methionine (A) or *l*-cystine (B). All supplements were added to the basal medium before autoclaving.

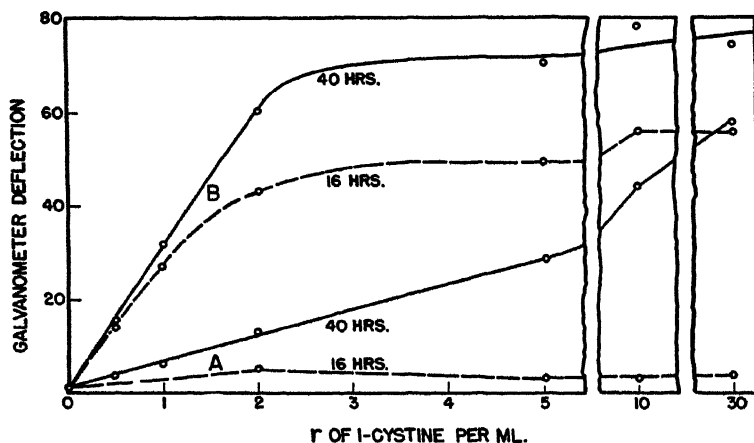


FIG. 3. The Response of Mutant 255-468 to *l*-Cystine in the Presence and Absence of Methionine.

All supplements were added to the basal medium before autoclaving. A. Un-supplemented basal medium; B. Basal medium with 10  $\gamma$  of *dl*-methionine/ml.

these mutants do not appear to be synthesizing methionine from the sulfur of  $\text{Na}_2\text{S}$  under these conditions. After transfer on the stock agar slants during a period of several months, mutant 255-468 was able to grow within 16 hours in the presence of cystine alone. A similar adaptation occurred during serial transfer in the presence of excess cystine. These strains were less stable than the original isolate and readily reverted to growth in the unsupplemented basal medium. This reversion was especially frequent in the presence of suboptimal concentrations of cystine.

#### *H<sub>2</sub>S Production by Escherichia coli and Mutant Strains*

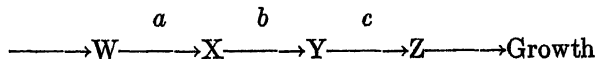
Four strains of *E. coli* (No. 15, K-12, B, and No. 2017) and mutants 508-462, 255-468, 532-171, and 3-301 were used and each organism was tested by two procedures. In one,  $\text{H}_2\text{S}$  production was determined during growth of the organisms in the basal medium containing the appropriate sulfhydryl compound. *dl*-Methionine was added at a concentration of 10  $\gamma$ /ml. when necessary for growth. In the other procedure a suspension of about one billion cells/ml. in the saline buffer was incubated with the sulfhydryl compound. Lead acetate papers were used to demonstrate  $\text{H}_2\text{S}$  formation. The results by the two procedures were qualitatively identical.

All of the organisms formed large amounts of  $\text{H}_2\text{S}$  in the presence of 50  $\gamma$  of added cysteine or cystine/ml. Positive tests were obtained with cysteine after 16 hours of growth or after 4 hours' incubation with the resting cells. Thus cysteine desulfhydrase (12) appears to be present in all of the strains tested, including those mutants which require added cysteine for growth. In the presence of 100  $\gamma$  of *dl*-homocysteine/ml. none of the organisms produced  $\text{H}_2\text{S}$  within 24 hours and only traces of  $\text{H}_2\text{S}$  were obtained after 2-3 days of incubation. *E. coli* No. 15 did not produce detectable quantities of  $\text{H}_2\text{S}$  within 24 hours with concentrations of 5-800  $\gamma$  of homocysteine/ml. The 4 strains of *E. coli* were transferred 4 times in the basal medium containing 10  $\gamma$  of *dl*-homocysteine/ml. and tested again. None of the strains produced  $\text{H}_2\text{S}$  from homocysteine at a significant rate. The traces of  $\text{H}_2\text{S}$  obtained from homocysteine after long incubation might well have been formed *via* cysteine. Thus homocysteine desulfhydrase does not appear to be present in significant amounts in any of the strains. This is in agreement with the results of Fromageot and Desnuelle (13).



*Tests for Syntrophism*

Heterocaryon formation or sexual crossings which are used to test the identity of related mutant strains of *Neurospora* (14) cannot be performed with *E. coli*. Another method of differentiation employed with *Neurospora* mutants is based on the production by one mutant of a precursor which will support the growth of another mutant which has a break at an earlier point in the same synthetic chain. If precursor Y in the chain



is stable and diffusible, filtrates of cultures of mutant *c* (the mutant with a break at reaction *c*) grown in the presence of limiting concentrations of Z will support the growth of mutants *a* or *b*. The turbidity attained by a mixed inoculum of mutant *c* and *a* or *b* with limiting concentrations of Z should also be greater than when either mutant is present alone. Negative results are not proof of identity, however, since Y may be unstable or nondiffusible. This phenomenon of increased growth with mixed inocula has been termed syntrophism (15).

In a typical test, inocula of mutants 1-344, 3-301, or 532-171, or of a combination of 1-344 and 3-301 reached about 15% of maximal growth in the presence of 1  $\gamma$  of *L*-methionine/ml. When mutant 532-171 and either mutant 1-344 or 3-301 were present, about 3 times as much growth was obtained. Thus syntrophism did not occur between strains 1-344 and 3-301 but did occur between mutant 532-171 and either of these strains. In the tubes containing both mutant 532-171 and mutant 3-301 the final population consisted almost entirely of cells which were able to utilize homocysteine, *i.e.*, mutant 532-171. Therefore, the syntrophism probably consisted primarily of the growth of mutant 532-171 on the precursor of methionine, probably homocysteine, formed by mutant 3-301.

The pertinent data are summarized in Table III. Syntrophism occurred between strains of different nutritional groups but not between strains of the same group. While the strains within the various groups appear to be identical, it should be emphasized that the absence of syntrophism is not proof to identity.

TABLE III  
*Syntrophism between Mutant Strains*

Mutant	Occurrence of syntrophism <sup>a</sup>	
	Present	Absent
932-230	1590-230	1251-171
1251-171	1590-230	932-230
1590-230	932-230	
	1251-171	
508-462	532-171	255-468
255-468	532-171	508-462
532-171(*)	Strains marked (+)	Other strains marked (*)
754M-171(*)	Strains marked (+)	Other strains marked (*)
282-460(*)	Strains marked (+)	Other strains marked (*)
495-460(*)	Strains marked (+)	Other strains marked (*)
654-228(+)	Strains marked (*)	Other strains marked (+)
3-301(+)	Strains marked (*)	Other strains marked (+)
1-344(+)	Strains marked (*)	Other strains marked (+)
1-273-384(+)	Strains marked (*)	Other strains marked (+)

<sup>a</sup> Each test was performed in triplicate. Tubes which were inoculated with two mutants received one-half of the usual inoculum of each. Limiting concentrations of *l*-cystine were present in experiments involving mutants 932-230, 1251-171, and 1590-230 and of *dl*-homocystine with mutants 508-462 and 255-468. *l*-Methionine was used in all other tests. Where syntrophism is indicated to be absent, the turbidity obtained with mixed inocula at 24 hours of incubation did not exceed that obtained with either of the single cultures. Where syntrophism is indicated to be present, increases of from 50-300% occurred with mixed inocula.

## DISCUSSION

The data of Table I show that mutants 1251-171 and 932-230, both of which are unable to utilize sulfate, vary in their ability to utilize cystathionine and methionine. The cystine-requiring strains 508-462 and 255-468 differ in their ability to grow with cystathionine or homocystine. Also, the 4 strains able to use homocystine grow at different rates with low homocystine levels. Since these differences occur between strains which apparently involve the same mutation, it is likely that the differences existed among the cells of the parent *E. coli* previous to the mutations described here.

Good growth of mutant 255-468 was observed occasionally after 2 or 3 days of incubation in the presence of 100  $\gamma$  of sodium thio-

glycollate/ml. Growth did not occur in the unsupplemented basal medium or in the presence of  $\text{Na}_2\text{S}$ . The cultures which had grown on thioglycollate grew within 16 hours on subculture in the presence of thioglycollate, cystine, or methionine, within 48 hours with  $\text{Na}_2\text{S}$ , and only at 72 hours in the basal medium. The cultures from  $\text{Na}_2\text{S}$  then grew within 16 hours on subculture with  $\text{Na}_2\text{S}$  and within 24 hours in the basal medium. The intermediate culture which was able to grow more rapidly with thioglycollate than with  $\text{Na}_2\text{S}$  may have been forming cystine and methionine from thioglycollate by a process which did not involve sulfide. The extreme instability of these cultures makes this uncertain, however. Cultures which had reverted (*i.e.*, which had regained the ability to grow in the unsupplemented basal medium) grew essentially at the same rate as did the parent *E. coli*. The reversion occurred then in a stepwise manner rather than as a sudden back mutation. The genetic basis of this process is obscure at present but adaptation to alternate synthetic mechanisms may possibly be involved.

In Fig. 4 are indicated some of the possible interconversions of sulfur compounds in *E. coli* and the probable point of the enzyme loss

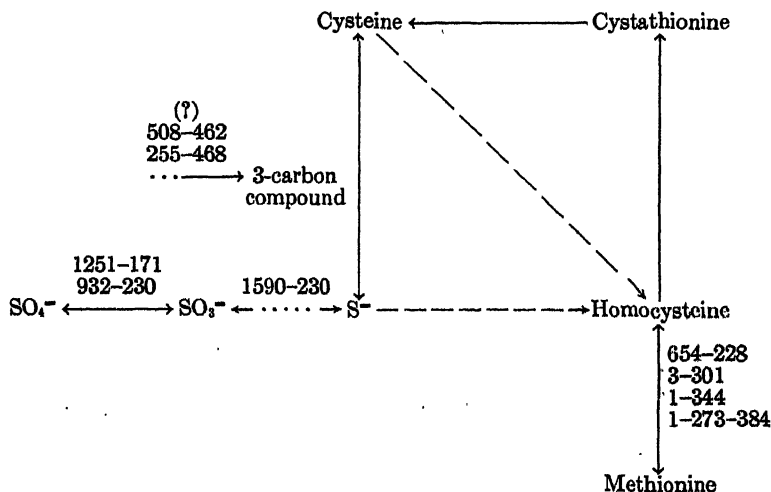


FIG. 4. Possible Interconversions of Sulfur Compounds in *Escherichia coli*.

The numbers of the mutants are placed beside the reaction which they cannot carry out.

(or break) in certain of the mutants. Thus mutants 932-230 and 1251-171 probably lack the ability to reduce sulfate to sulfite, whereas mutant 1590-230 has lost some reaction involved in the further reduction of sulfite. The mutants which are unable to grow with homocysteine appear to lack the ability to convert this compound to methionine. Since 4 other mutants were able to perform this conversion, it seems logical to assume that this conversion is the site of the mutation in these strains rather than that other methylating processes or additional growth factors are normally involved (16). Our data do not indicate the exact mechanism of this conversion, however. The fact that the loss of ability to utilize homocysteine produces a methionine deficiency is good evidence that homocysteine is a precursor of methionine in the parent *E. coli*.

The presence of cysteine desulphydrase in *E. coli* suggests that cysteine may normally be synthesized by the reverse of this reaction. If this is the situation, mutants 508-462 and 255-468 must be unable to synthesize the sulfur-free molecule required for the reaction since they still possess the desulphydrase. Cystathionine replaces cysteine, at least in part, for all mutants requiring cysteine. It is generally more active than are homocysteine or methionine. Thus cysteine may be formed from the latter compounds *via* cystathionine.

The point of the enzyme loss in the mutants which are unable to synthesize homocysteine cannot be placed at present. The absence of homocysteine desulphydrase indicates that this molecule is not formed by the direct addition of  $H_2S$  to the requisite sulfur-free molecule. The conversion of homocysteine to cysteine *via* cystathionine might be reversible in *E. coli* although it is apparently not reversible in the rat (17). If the normal synthesis of homocysteine by *E. coli* occurs *via* cysteine and cystathionine the break with these mutants must be in the formation of homocysteine from cystathionine since the latter compound cannot replace methionine for growth. No alternate mechanisms for the conversion of the sulfur of  $Na_2S$  or cysteine to homocysteine can be suggested at present.

#### ACKNOWLEDGMENT

We wish to thank Mrs. Florence Mercer for assistance in the isolation of some of the mutant strains and Miss Aileen B. Perkins for her aid in testing their growth requirements.

## SUMMARY

A series of mutant strains of *Escherichia coli* are described, all of which are unable to grow with sulfate as their only source of sulfur. Mutants 932-230 and 1251-171 are able to utilize sulfite, whereas mutant 1590-230 requires sulfide or other reduced forms of sulfur. Mutants 508-462 and 255-468 are unable to synthesize cysteine. Four strains require the addition of homocysteine or methionine for growth and another group can utilize only preformed methionine. Those strains which require reduced forms of sulfur or cysteine can use a portion of their sulfur requirements more efficiently in the form of methionine.

*E. coli* produces  $H_2S$  readily from cysteine but does not form  $H_2S$  from homocysteine even after four transfers in its presence.

The site of the enzyme loss in certain of the mutants is indicated, and the possible interconversions of the various sulfur-containing substances in *E. coli* are discussed.

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# The Production of Exocellular Pectic Enzymes by *Penicillium chrysogenum*. I. On the Formation and Adaptive Nature of Polygalacturonase and Pectinesterase

H. J. Phaff

*From the Food Technology Division, University of California, Berkeley, California*

Received May 27, 1946

## INTRODUCTION

Soluble pectic substances can be hydrolyzed enzymatically by the combined action of pectinesterase (PE) and polygalacturonase (PG) (1,2). Pectinesterase hydrolyzes methanol from the esterified carboxyl groups of galacturonic acid units in pectin. These anhydrogalacturonic acid units are arranged in long straight chains of high molecular weight. They are in the pyranose form and connected to each other by 1,4- $\alpha$ -glycosidic linkages. The latter undergo hydrolytic cleavage by polygalacturonase.

PE occurs practically free of PG in varying quantities in the leaves, roots and fruits of numerous higher plants. In fungi having pectolytic activity, however, both seem to occur. Thus, for example, in commercial preparations of pectic enzymes obtained from fungi, PG is always accompanied by PE. Only recently satisfactory methods for separating PE from PG have been reported. McColloch and Kertesz (3) were able to remove PE from commercial pectinase preparations by a cation exchange resin at the proper pH. Jansen and MacDonnell (1) essentially freed PG from PE by several acid treatments (pH 0.6) followed by precipitation with ammonium sulfate between treatments. They also showed that only de-esterified portions of pectic substances were attacked by PG. This necessitates the use of pectic acid as a substrate for quantitative PG determinations, because the possibility exists that methanol hydrolysis by PE of the pectinase complex will be the limiting factor in the rate of glycosidic hydrolysis of pectin by PG. Unfortunately, when the experimental work described in this paper was carried out, the work by Jansen *et al.* had not been published and pectin was used to test for PG and PE both. Recently, however, *P. chrysogenum* was grown again on a pectin medium and PG and PE activity determined using the quantitative methods of assay as described in (1). The relatively large proportion of PE in the pectinase complex of this fungus indicates that the use of pectin for PG assay has at least qualitative value.

Relatively little information is available as to the conditions under which fungi produce pectic enzymes. Ehrlich (4) routinely grew *Penicillium ehrlichii* on a yeast extract medium containing 2% citrus pectin and malt extract at a concentration of 2° Balling. The mycelium was ground and the extract combined with the culture medium before alcohol precipitation of the pectinase complex. PE always seems to accompany PG in *P. ehrlichii* preparations. Harter and Weimer (5) found that the pectinase complex is produced by *Rhizopus tritici* only when grown on various vegetable decoctions, or in synthetic media containing pectin as a carbon source. The enzyme was not formed in a synthetic medium with glucose as a source of carbon or in beef bouillon. Both intracellular and extracellular pectic enzymes were produced on sweet potato decoction. Harter and Weimer found that the maximum enzyme content of the hyphae and the solution was attained in about 24 and 48 hour cultures, respectively. Harter and Weimer in testing several other *Rhizopus* species, found that mycelium and culture medium of *R. nigricans* and *R. artocarp*i (both of which are parasitic on sweet potato) contain only relatively small amounts of pectic enzymes. However, *R. chinensis* and *R. microsporus* (two non-parasitic species) retain only small amounts of enzyme in the mycelium, but secrete comparatively large quantities into the culture solution. Other species formed about equal amounts in mycelium and culture liquid.

Willaman and Kertesz (6) grew *Penicillium glaucum* Link in media containing, in addition to a standard salt supplement, various combinations of sucrose, starch, peptone and pectin. Qualitative enzyme tests based on the time necessary to clarify grape juice, did not reveal any stimulatory action of pectin, but the experiments indicated that apparently the presence of sucrose was important. In most instances the quantity of exo- and endocellular pectic enzymes was approximately the same.

Proskuriakov and Ossipov (7) used the ground, dried mycelia of the following molds as sources of pectinase: *Aspergillus oryzae*, *A. niger*, *Mucor* sp., *Botrytis cinerea*, *Sclerotinea fructigena*, and *Rhizopus nigricans*. They demonstrated that addition of pectin to the medium markedly increased the formation of pectic enzymes. Of two strains of *A. oryzae*, one proved to be 4 times as active as the other when grown under identical conditions. This indicates that it is the strain rather than the species which is important in selecting an organism for the production of enzymes. In all cases, they observed that a drop in pH and an increase in reducing power accompanied the action of polygalacturonase on pectin. The more active the PG action, the greater the drop in pH. This would indicate that the more active the PG was, the more pectin-esterase was present in the various mold preparations. They also showed that, when calcium pectate can no longer be obtained after alkali treatment of a test pectin solution under the influence of the pectinase complex, a further increase in reducing substances occurs as a result of continued action of PG.

Such limited investigations on the formation of pectic enzymes by fungi as have been reported deal largely with studies of endocellular enzymes, and the more commonly used commercial preparations of fungal pectic enzymes are obtained from the mycelial mats, as well as the culture fluids. When the author investigated pectic enzyme forma-

tion by a number of fungi, a strain of *Penicillium chrysogenum* was found to secrete active pectic enzymes in the culture medium. This organism was selected for detailed investigation since, through its use, it is possible to define more clearly the conditions under which pectic enzymes are elaborated and to obtain purer pectic enzymes. The formation and adaptive nature of the PG and PE produced by this fungus are discussed in this paper.

## METHODS

The strain of *P. chrysogenum* used was found to grow well in a synthetic medium consisting of tap water with 0.1% ammonium nitrate, 0.1%  $\text{KH}_2\text{PO}_4$  and 0.05% magnesium sulfate in addition to a source of carbon. Strong organic acids, if used as carbon sources, were partially neutralized with NaOH to pH 3.2. In this work, only standing cultures of the fungus were used. Growth took place at room temperature (approximately 22–25°C.) unless otherwise specified. The cultures were inoculated with a spore suspension obtained from a week-old slant culture of the fungus on potato-agar-dextrose in 24 mm. test tubes. The suspension was made by gently rubbing an inoculating loop over the agar surface which was flooded with sterile water. The ratio between mycelial surface to volume of medium is indicated for each particular set of experiments. The reducing values of solutions were determined by Schoorl's iodide method with Soxhlet's solution (8). Pectic acid was determined as calcium pectate according to the method of Carré and Haynes (9). Methyl alcohol was determined colorimetrically, after distillation, by von Fellenberg's method (10); bound methanol was first hydrolyzed by alkali. The PE activity was measured by determining the amount of methanol liberated from a test pectin solution at a certain temperature at various time intervals (Sloep (11)). PG activity was usually measured by determining the increase in reducing power of a test pectin solution over a definite period of time at a certain temperature. Jansen and MacDonnell (1) showed that, at least during the initial stages of hydrolysis, the increase in reducing power is a linear function of time. In all cases the pectin used for activity determination was a 2% 160 grade citrus pectin, dissolved in a *M*/8 phosphate buffer at pH 3.7. Methanol content of the air dry pectin was 7.9%. Although the pH used was the optimum for PG, it was somewhat lower than the optimum for PE of this fungus (4.3–4.4). However, the PE activity was found to be only about 10% lower at pH 3.7 than at its optimum pH.

In certain cases, for reasons of speed and simplicity, a modified Mehltz and Scheuer method (12) was used for rough comparison of the activities of different cultures. The method is based on measuring very rapid drop in viscosity of a test pectin solution under the influence of the pectic enzymes. This physical change invariably precedes the main chemical changes in such a solution. This method can be used as an index only of pectinase activity (changes resulting from the combined action of PG and PE), since the change in viscosity does not parallel the increase in reducing value resulting from the hydrolysis (see Kertesz (13)).



The determinations were made in an Ostwald viscosimeter at 25°C. Ten cc. of a 1.5% citrus pectin solution in a phosphate buffer at pH 3.4 were mixed with 1 cc. of the enzyme solution to be tested. The initial viscosity of this mixture (to which heat-inactivated enzyme had been added) was measured as a control. The reaction was allowed to proceed for exactly 30 minutes and the viscosity again determined. The decrease in viscosity expressed in *per cent* of the total possible decrease, was taken as a rough measure of enzyme activity. The total possible decrease was found by subtracting the flow time (in seconds) of a 1.5% galacturonic acid solution in phosphate buffer from that of the original pectin solution.

## RESULTS

When grown on a mineral medium containing pectin as a source of carbon, this strain of *P. chrysogenum* starts producing exocellular pectic enzymes as soon as the surface mat begins to form. The secretion continues even after autolysis of the mycelium supersedes the formation of new hyphae. The latter is evidenced when mycelial weight determinations in a series of flasks show a drop after reaching a maximum value. This is illustrated in Table I. Intracellular pectic enzymes could not be found in the mycelial mats except in very small quantities.

TABLE I

*Relation between Fungus Growth and Amount of "Pectinase Complex"  
Secreted in 125 cc. Erlenmeyer Flasks Containing 30 cc. of  
Mineral Pectin Medium*

Age of culture in days	Weight of dry mycelium in mg.			Activity of culture fluid in <i>per cent</i> viscosity drop		
	1	2	Average	1	2	Average
2	17.3	17.3	17.3	5.8	6.8	6.3
3	48.5	52.8	50.7	37.0	38.5	37.8
4	83.3	88.0	85.7	65.6	70.0	67.8
5	107.7	116.0	111.9	80.7	83.3	82.0
7	115.2	115.6	115.4	84.4	85.5	84.9
8	115.4	108.5	111.9	87.0	85.9	86.4
9	103.8	108.7	106.3	85.5	88.1	86.8
12	99.7	102.2	100.5	91.0	91.4	91.2
13	97.9	100.0	98.9	91.0	91.8	91.4
14	98.9	99.5	99.2	91.0	92.4	91.7

In another experiment, carried out to determine how long a certain mycelial deck is able to continue pectinase production in the presence of sufficient substrate, the enzyme-containing culture liquid was removed aseptically and replaced by a fresh

pectin medium of the same original composition, without disturbing the mycelial mat. This process was repeated 3 times, the pectinase activity of the culture liquid being determined each time by the viscosity method. It was noticed that the fungus deck became thinner and more fragile during each replenishing operation, but the total activity in each batch was about the same. In one experiment, in which a gallon bottle was used containing one liter of the above pectin medium, the activity figures were: 85.0 (14 days), 80.0 (21 days), 82.4 (21 days), 80.0 (27 days). Total age of mycelium, 83 days at the close of the experiment.

The exocellular pectic enzymes, as well as the living fungus itself, contribute to the changes taking place in the medium (see Table II). The data, which are typical of many similar experiments, show that PG activity progressively increases for a time, but eventually begins to decline; in this case, a small drop occurs after 19 days. The latter may be explained by the considerable rise in pH, for the stability of PG of this fungus decreases at high pH values.

TABLE II  
*Changes in Culture Fluid during Growth of P. Chrysogenum*

Time of growth in days	Pectinase activity in per cent viscosity drop of a test pectin solution	Reducing power /10 cc. culture liquid in cc. 0.1 N thio-sulfate	mg. Ca-pectate /10 cc. of culture liquid	pH
0	0	8.04	143.2	2.83
2	—	7.35	142.2	2.79
3	4.9	8.06	121.0	2.59
4	23.2	18.91	0.0	2.45
5	40.7	23.03	—	2.42
7	58.1	19.10	—	2.50
9	80.0	12.83	—	2.73
11	86.5	8.34	—	2.95
14	90.6	3.76	—	3.35
18	92.4	0.52	—	5.58
19	88.1	—	—	6.10

(2 liters of mineral medium with 2% commercial 160 grade citrus pectin in a 6 liter Erlenmeyer flask)

Since purified pectin reduces Fehling's solution only very slightly, the initial reducing power is due to glucose normally present in commercial pectins. An initial small drop in reducing power indicates that the fungus multiplies for a while at the expense of the glucose. This conclusion is confirmed by the fact that another *Penicillium* species,

which was unable to attack pectin, would utilize the glucose present in commercial pectin, thereby diminishing the reducing value to a very small figure, typical for pure pectin.

Column 2 shows that by the third day a small amount of polygalacturonase has formed in the medium, which causes a corresponding small increase in reducing power by glycosidic hydrolysis of the pectin chain. When more polygalacturonase is formed this process is accelerated. The final product of hydrolysis is galacturonic acid, but this also serves as a carbon source and is utilized, thus accounting for the final loss in reducing power of the culture fluid. In the fourth column is given the amount of calcium pectate obtained from 10 cc. of culture liquid after splitting off the methyl alcohol by means of cold, dilute NaOH. After the fourth day no more precipitate is obtained, although the reducing power has not reached a maximum. The molecular size of the smallest polygalacturonide capable of forming a precipitate with calcium is not known, but these data may be construed to indicate that at least the digalacturonide will not form an insoluble calcium salt.

In the last column the changes in pH values of the culture fluid are reported. The initial drop in pH is due to at least 3 factors. First, the fungus produces PE, which hydrolyzes off the methyl alcohol groups, at the same time liberating a corresponding quantity of carboxyl groups. Secondly, the mold secretes PG, which hydrolyzes polygalacturonic acid into monogalacturonic acid and the latter is more strongly dissociated than the former. Thirdly, the organism utilizes the ammonium ion more easily than the nitrate ion and therefore increases the acidity.

The final hydrogen ion concentration in the culture fluid depends largely on the source of nitrogen used. This is illustrated in Table III, where a few nitrogen sources are compared with regard to their suitability in furnishing the nitrogenous constituents for polygalacturonase. The total nitrogen content in all cultures was 350 mg. N/l.

The data in Table III indicate that ammonium nitrate and ammonium sulfate are equivalent as nitrogen sources for PG production. Both compounds are acid-forming when their ammonium part is being utilized, but the former less than the latter because the nitrate ion will also be utilized slowly.  $\text{KNO}_3$  is less suitable than the former two, probably because the nitrogen is in a higher oxidation state. Furthermore, the accumulation of basic potassium as carbonate causes the

medium to become alkaline more rapidly, with subsequent deterioration of the pectinase.

Both of the amino acids are rather good sources of nitrogen; but since they do not possess an acid-forming anion, here, too, the medium becomes alkaline as soon as the galacturonic acid is used up.

TABLE III

*Penicillium chrysogenum* in Tap Water with 1.5% Commerical Citrus Pectin,  
0.05%  $KH_2PO_4$ , 0.05%  $MgSO_4$  and Various Nitrogen Sources

(Activity referred to is of the pectinase complex)

N-source	6 days of growth		16 days of growth	
	pH	Activity in per- cent viscosity drop	pH	Activity in per- cent viscosity drop
0.1% $NH_4NO_3$	2.25	69.0	3.20	87.8
0.25% $KNO_3$	2.75	49.5	7.70	27.4
0.165% $(NH_4)_2SO_4$	2.28	74.0	2.50	88.1
0.188% Glycine	2.90	61.0	7.56	58.8
0.198% DL-alanine	2.85	53.0	7.66	64.3

Ammonia nitrogen, therefore, is a superior source for PG production. The presence of nitrate is not particularly desirable, but it has no harmful effect.

It seemed of considerable interest to determine what compounds would stimulate the production of pectic enzymes. A number of organic compounds, listed in Table IV, were tried as sources of carbon in the above mineral medium. The results show that in addition to pectin and pectic acid, D-galacturonic acid, mucic acid, L-galactonic acid,\* and gum tragacanth are all suitable carbon sources for pectinase (PG + PE) formation. All the other compounds supporting growth, however, only stimulated the production of traces of activity at most. Although D-galacturonic acid, mucic acid and L-galactonic acid are suitable substrates, other compounds, including closely related ones, such as D-galactose, D-galactonic acid, dulcitol, L-galacturonic acid and L-arabinose produce but traces of activity although they support a good fungus growth. L-arabinose (naturally occurring isomer) may be looked upon as a decarboxylated derivative of D-galacturonic acid.

\* Kindly furnished by Dr. H. S. Isbell.

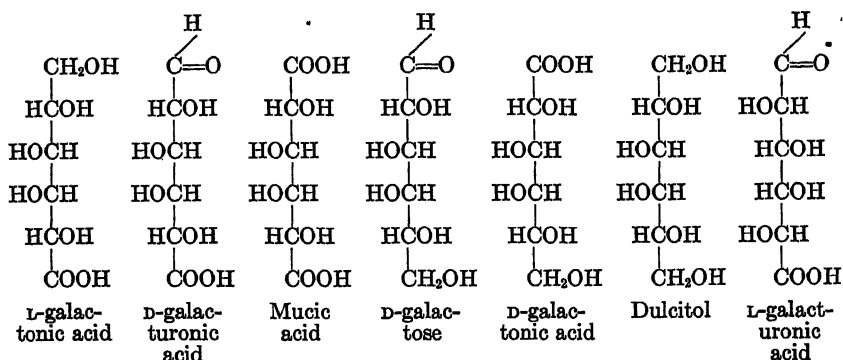


TABLE IV

*Pectinase Production by P. chrysogenum in Standing Cultures with a Variety of Carbon Sources*

Name of compound tested	Description of growth	Activity
1. L-arabinose	excellent	absent or trace
2. D-arabinose	fair	absent
3. D-xylose	excellent	absent
4. D-glucose	excellent	trace
5. D-fructose	excellent	trace
6. D-mannose	excellent	absent
7. D-galactose	excellent	absent
8. α-Methylglucoside	excellent	trace
9. Sucrose	excellent	absent
10. Lactose	excellent	absent
11. Maltose	excellent	trace
12. Gluconic acid	excellent	absent
13. 5-Ketogluconic acid	excellent	absent
14. D-galactonic acid (γ-lactone)	excellent	absent
15. L-galactonic acid (γ-lactone)	fair	excellent ✓
16. D-galacturonic acid	excellent	excellent ✓
17. L-galacturonic acid	slow start, later fair	absent
18. D-glucuronic acid	excellent	absent
19. Borneol glucuronic acid	no growth	—
20. Lactic acid	excellent	trace
21. Sodium pyruvate	excellent	absent
22. Hydroxyacetic acid	no growth	—
23. Glycerin	excellent	trace
24. Ethylene glycol	no growth	—
25. Propylene glycol	no growth	—
26. Dulcitol	good	absent

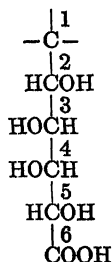
TABLE IV (Continued)

*Pectinase Production by P. chrysogenum in Standing Cultures with a Variety of Carbon Sources*

Name of compound tested	Description of growth	Activity
27. Dihydroxyacetone	excellent	absent
28. Glyceraldehyde	excellent	absent
29. Oxalic acid	no growth	—
30. Malonic acid	no growth	—
31. Succinic acid	excellent	absent
32. Adipic acid	excellent	absent
33. Maleic acid	no growth	—
34. Tartronic acid	fair	absent
35. D-tartaric acid	good	absent
36. DL-tartaric acid	good	absent
37. meso-Tartaric acid	good	absent
38. Malic acid	excellent	absent
39. Saccharic acid	excellent	trace
40. Citric acid	excellent	absent
41. Mucic acid	fair	excellent ✓
42. Dilute agar solution	no growth	—
43. Agar hydrolyzate	good	absent
44. Dextrin	excellent	absent
45. Pectin	excellent	excellent ✓
46. Pectic acid	good	excellent ✓
47. Tannic acid	good	absent
48. Alginic acid	fair	absent
49. Gum ghatti	good	absent
50. Gum tragacanth	excellent	excellent ✓
51. Gum arabic	excellent	absent
52. D-ascorbic acid	fair	absent

In explaining why only the first 3 of the above 7 compounds will support pectinase formation, two possibilities must be considered. First, pectinase formation may be stimulated by a particular molecule or part of such a molecule. Secondly, the stimulation may be due to some intermediate breakdown product. The latter alternative is least likely, since such intermediate products of metabolism would be common to more compounds than the 3 acids in question. It still remains possible that mucic acid is the only stimulatory compound and that L-galactonic acid and D-galacturonic acid undergo oxidative dissimila-

tion by the mold *via* the mucic acid stage. The three "active" acids have in common the structure depicted below.



To determine how many of the carbon groups in the above structure are essential, a number of compounds were included among those listed in Table IV, possessing one or more groupings identical with those shown in the last formula. Thus, tartronic and L-malic acids have carbon atoms 5 and 6 with identical stereochemical side groupings. L-tartaric acid possesses carbons 4, 5 and 6 and D-arabonic acid, those numbered 3, 4, 5 and 6. None of these compounds, in spite of excellent growth, produced more than traces of pectinase. Since it seems immaterial, in the mechanism of PG formation, whether the carbon atom in position 1 of the numbered formula carries an acid, aldehyde or alcohol group the presence of a primary alcoholic group rather than an aldehyde or carboxyl group in position 1 in D-arabonic acid in all probability would not prevent pectinase formation, and the conclusion seems justified, that the complete structure shown in the last formula is an essential part of the molecule responsible for enzyme formation.

D-galactonic acid resembles the above configuration, but its four secondary alcoholic groups plus carboxyl group are in a mirror image position compared with those of D-galacturonic acid. In L-galactonic acid (formed by reduction of D-galacturonic acid, see Isbell, 14), on the other hand, these groupings are in the same stereochemical configuration as in D-galacturonic acid. Since D-galactose does not stimulate PG production, it would appear that the combination of an aldehyde group in position 1, and the four secondary alcoholic groups on carbon atoms 2 to 5 of this sugar (which are identical with those in D-galacturonic acid) do not play an essential role in the specificity, although the same 4 alcoholic groups are necessary in combination

with a carboxyl group in position 6. The fact that the aldehydic group is not involved in the specificity is confirmed by the fact that mucic acid and L-galactonic acid, which are devoid of aldehydic groups, do stimulate pectinase formation.

It seemed of general interest to compare, for example, mucic acid and D-galacturonic acid on a quantitative basis with regard to their ability to stimulate pectic enzyme formation. The following experiment was performed to test the two compounds (see Table V).

TABLE V  
*PG and PE Production in Equimolecular Solutions of Mucic Acid and D-Galacturonic Acid*

Substrate and concentration			Activity of pectinase complex expressed as increase in reducing power of a test pectin solution in 9 hrs. at 23°C.				PE activity in mg. CH <sub>2</sub> OH hydrolyzed /100 cc. of a test pectin solution in 2 hrs. at 40°C. (pH 3.5) by 20 volume-% of culture liquid
D-glucose	D-galacturonic acid	Mucic acid	3.5 days	4.5 days	7.5 days	17 days	17 days
<i>M</i>	<i>M</i>	<i>M</i>					
0.020	0.000	—	0.1	0.05	0.15	0.29	nil
0.015	0.005	—	0.70	3.20	3.85	4.07	46
0.010	0.010	—	1.80	3.15	3.95	4.07	41
0.005	0.015	—	2.30	4.33	5.00	5.14	67
0.000	0.020	0.000	2.65	4.70	6.80	6.94	81
0.015	—	0.005	1.50	2.35	3.00	3.44	21
0.010	—	0.010	2.00	2.80	4.10	4.41	40
0.005	—	0.015	2.20	3.20	4.25	5.63	63
0.000	—	0.020	0.20	2.30	4.65	6.35	74

A series of 50 cc. Erlenmeyer flasks, containing 25 cc. medium, was inoculated with 1 cc. of a spore suspension of the fungus. The total substrate concentration was 0.020 *M* in all cases. One series consisted of mixtures of glucose and mucic acid, the other of mixtures of glucose and D-galacturonic acid. The mineral composition was 0.1% ammonium sulfate, 0.05% monopotassium phosphate and 0.05% magnesium sulfate. One cc. samples were taken periodically and tested for PG and PE activity. Due to the fact that growth was not equally rapid in all flasks, the figures showing the activities of the mucic and galacturonic acid solutions in the early stages of growth are not strictly comparable. However, the final figures indicate clearly that the two compounds studied are equal in their ability to stimulate PG and PE production.



The slight differences in the corresponding final activity figures are due to experimental error, since every row in Table V represents a different Erlenmeyer flask with small variations in total amount of growth. The values in the second row are almost the same as those in the third row and it is most likely that accidentally the composition of the medium in the second row was similar to that of the third row. Finally one may notice that the total amount of enzyme produced increases when more of the specific substrate is available, although the total substrate concentration is constant. The results present additional evidence that the two compounds have a common factor (perhaps mucic acid itself) which plays a role in enzyme production.

It remains to be explained why tragacanth gum is the only other suitable substrate for pectinase production. Ehrlich (15) first showed indirectly the presence of D-galacturonic acid in tragacanth gum hydrolyzate and Weinmann (16) later isolated and identified the acid. Von Fellenberg (10) showed the presence of methyl alcohol in gum tragacanth. The scanty information available on the structure and composition of gum tragacanth is discussed by Norman (17). Since the fungus grows well on this gum its content of D-galacturonic acid is undoubtedly responsible for pectinase production.

Two obvious experiments suggest themselves now. First to determine the action of a pectin culture liquid on gum tragacanth and second, the action of the tragacanth culture liquid on the same substrate. Surprisingly enough it was found that PG and PE from a pectin culture liquid had almost no effect on a gum tragacanth solution if methyl alcohol liberation and increase in reducing power were taken as criteria. The action of its own culture liquid was faster, but very slow as compared with the action of a pectin culture liquid on pectin. This indicates that the fungus when growing on gum tragacanth forms a small amount of a specific enzyme able to hydrolyze the gum slowly. When galacturonic acid is liberated the fungus rapidly utilizes this acid with resultant production of PG and PE.

The almost complete lack of gum tragacanth hydrolysis by PG and PE from a pectin culture liquid may be explained by assuming a different bonding between the galacturonic acid units in gum tragacanth. Furthermore, according to Norman, the galacturonic acid residues are united with an equal number of arabinose units, which may account for the retarding effect on the rate of hydrolysis.

It is also noteworthy that L-galacturonic acid and other uronic acids such as D-glucuronic acid and D-mannuronic acid (present in alginic acid) have no effect on pectinase formation.

Most attention thus far has been devoted to the behavior of PG, but cultures of *P. chrysogenum* growing on a pectin-containing medium produce PE in addition to PG. This is evidenced by the fact that such a culture liquid has a strong hydrolytic effect toward esterified  $\text{CH}_3\text{OH}$  in pectin. Since it is difficult to separate PG and PE by chemical or physical means it was hoped that this could be effected by biological means. PE is not a constitutive enzyme of this fungus since in no case in which PG was absent with a certain growth substrate (Table IV) was PE activity found. This would have been detected by gelation of the test pectin solution (Ca-pectinate formation) as occurs when, for example, the juice of clover leaves is added. Such a leaf extract is rich in PE. Since the esterase is not constitutive, but apparently adaptive (it is formed with pectin as a substrate), the possibility existed that D-galacturonic acid, mucic acid and L-galactonic acid would produce PG free of the esterase, the three compounds being devoid of methyl ester groups. When this was tested the result was that pectin, D-galacturonic acid, mucic acid and L-galactonic acid were all active and roughly equivalent in their ability to stimulate PE formation. Similarly to PG the amount of PE increased when more of the specific substrate was present. The results of the latter experiment are given in the last column of Table V.

It must be concluded, therefore, that PE, like PG, is not a constitutive enzyme but, as in the case of PG, its production is conditioned by the presence in the medium of certain structural groupings, which happen to be the same as those necessary for the formation of PG. Of course, D-galacturonic acid must be considered essentially the product of hydrolysis of both PG and PE action (although through a different mechanism) and mucic acid and L-galactonic acid apparently possess the required specific stereochemical configuration necessary for the formation of both pectic enzymes. Thus, the two mold-produced enzymes are adaptive and depend for their formation on the same "activating structure" in the medium. The results reported in Table IV apparently support Yudkins (18) "mass action" theory of enzyme formation. He postulates a steady state between the adaptive enzyme involved and some precursor. In most compounds tested it was possible

to measure very small quantities of polygalacturonase. The combination of the enzyme with any suitable substance (in this case D-galacturonic or mucic acid) would result in a disturbance of the steady state and more enzyme would be formed from the precursor to restore the prior conditions. Yudkin presupposes that the products of enzyme action as well as the substrate stimulate its production. This, for example, has been shown for invertase (19) and is true in our case. The data available for our fungal PG and PE indicate that the products (galacturonic acid) apparently have greater stimulating power than the substrate (pectin).

#### ACKNOWLEDGMENT

The author is indebted to Dr. M. A. Joslyn and Dr. H. A. Barker for advice during the investigations and for reading the manuscript.

#### SUMMARY

1. A strain of *Penicillium chrysogenum*, able to grow well in a synthetic medium, was used as a source of pectolytic enzymes, which are secreted by this mold into the culture medium. No more than traces of endocellular polygalacturonase could be demonstrated. The relation between fungus growth and amount of pectinase complex secreted is illustrated.

2. The utilization of pectin was followed in standing growing cultures by determining chemical changes in the medium.

3. A number of organic compounds were tested as carbon sources for pectic enzyme formation. It was shown that polygalacturonase and pectinesterase are both adaptive enzymes (although not in a strict sense), their formation being conditioned by the presence of either pectin, pectic acid, D-galacturonic acid, mucic acid, L-galactonic acid or gum tragacanth in the medium. No other compounds tested exerted this stimulatory action. PG and PE behave identically with respect to the dependency of their formation on certain structural groupings in the substrate. The role of gum tragacanth in supporting enzyme formation is discussed.

4. Ammonium salts proved to be more suitable than nitrates and some amino acids as a nitrogen source for pectic enzyme production.

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# The Oxidation of Carbohydrates by a Surface Strain of *Penicillium notatum*

Frederick T. Wolf

*From the Department of Biology, Vanderbilt University, Nashville, Tennessee*

Received September 9, 1946

## INTRODUCTION

Shortly after penicillin was discovered by Fleming, it was found that this antibiotic was produced by *Penicillium notatum* when the fungus was grown in a solution of inorganic salts (a modified Czapek-Dox solution) with glucose as the only source of carbon (2). This was the medium employed during the early studies of the Oxford group of investigators (1), but lactose-corn steep media later became the basis of all large-scale commercial production (3, 7a, 7b).

A great deal of attention has been devoted to variations in the ingredients of culture media in relation to the yield of penicillin. Although Dimond and Peltier (4) have studied the utilization of several sugars by *P. notatum* in relation to control of pH of the cultures, and other workers (6, 7a, 7b) have devoted considerable attention to certain specific carbohydrates, no extensive study of the role of carbohydrates in the metabolism of *P. notatum* has been made. It would appear that respirometer techniques might be particularly well suited to a determination of the abilities of *P. notatum* to respire various carbohydrates, and that the results of such a study might be applicable to certain of the problems encountered in commercial penicillin production. Accordingly, the present studies were conducted, using a surface strain of *P. notatum*. The recent development of superior submerged strains, which produce yields of penicillin much greater than are possible in surface culture, would appear to lessen somewhat the value of this investigation.

## MATERIALS AND METHODS

The strain of *P. notatum* used in these experiments was obtained from the Squibb Institute for Medical Research in 1943, and has been designated NRRL 1249 by workers at the Northern Regional Research Laboratory. According to Moyer and Coghill (7a), it is capable of producing 160–220 Oxford units per ml. of penicillin under suitable conditions.

The medium employed was the "Improved Medium" of Moyer and Coghill (7a) which has the following composition:  $\text{NaNO}_3$ , 3.0 g.;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.25 g.;  $\text{K}_2\text{HPO}_4$ , 0.5 g.; glucose monohydrate, 2.75 g.;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.044 g.;  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 0.004 g.; lactose monohydrate, 44.0 g.; and corn steep liquor (55% solids), 100 g. per liter. Erlenmeyer flasks of 125 ml. capacity, containing 50 ml. of the corn steep medium, were inoculated from stock cultures of the organism, and incubated at room temperature.

The experimental procedures utilized a Fenn microrespirometer of the differential type, consisting of two identical glass vessels of 15 ml. capacity connected by a capillary containing an index droplet of kerosene (5). KOH wells within each vessel provided for the absorption of  $\text{CO}_2$ . The apparatus was placed in a water bath, and shaken by a motor-driven eccentric at the rate of 73 cycles (146 movements)/min., through a horizontal distance of 40 mm., to provide for equilibration of temperature and pressure. The temperatures at which the various experiments were performed ranged from 19.8° to 24.2°C., but remained constant to within  $\pm 0.1^\circ\text{C}$ . during any single experiment. The buffer used was *M/20* Sørensen's phosphate, having a pH of 7.3. The various carbohydrates tested were of C. P. or reagent grade, and were Eastman, Difco or Pfanstiehl products.

In the experiments dealing with carbohydrates, 0.3 ml. of 10% KOH was placed in the center well of each respirometer vessel. The mycelia were suspended in 2.0 ml. of Sørensen's phosphate buffer within the experimental vessel, buffer alone being present in the control vessel. As a result of oxygen consumption by the mycelia in the experimental vessel, the index droplet in the capillary connecting the two vessels moved toward the experimental vessel, the rate of movement being taken as a measure of oxygen consumption. Following equilibration of temperature and pressure, the stopcocks were closed to the outside, and the oxygen consumption was measured at 5 minute intervals for a period of 40 minutes. The buffer was then removed from both vessels and replaced with an equal volume of a *M/10* solution of the particular carbohydrate to be tested (dissolved in *M/20* Sørensen's phosphate buffer). The oxygen consumption of the same mycelia was then determined during a second 10 minute period. The time between the control and experimental determinations did not exceed 10 minutes. The difference between the oxygen consumption in the control and experimental determinations was taken as a measure of the effect of the carbohydrate added.

At the completion of each experiment, the mycelia were removed from the respirometer vessel, placed upon a tared watch glass, and dried to constant weight in a desiccator containing Drierite. The  $\text{Q}_{\text{O}_2}$  was calculated from the dry weights of the mycelia, the oxygen consumption (in mm. of capillary/hr.) and a calibration of the volume of the capillary/unit length.

The experiments with carbohydrates were performed using mycelia which had grown upon the corn steep medium for 6 days. This period has been found to be sufficient for the development of a thick hyphal mat with abundant sporulation upon the surface of the culture solution, and the penicillin yield is usually near maximal at this time. Six days after inoculation the mycelia were removed from the corn steep medium and allowed to deplete their food reserves by being placed in distilled water for 20-24 hours prior to the experiments. Supplementary methods required for other purposes will be described in connection with the results.

## RESULTS

Data concerning pH changes in the culture media and dry weights of mycelia produced were obtained in several series of cultures. These results are in close agreement with those of Moyer and Coghill (7a), and hence are not presented in detail.

Apparently no information is available concerning the oxygen consumption of *P. notatum* in surface cultures, although this matter has been briefly considered in studies by Koffler *et al.* (6) dealing with submerged fermentations. A series of experiments was therefore conducted in which the oxygen consumption of mycelia of different ages was measured in the corn steep media in which they grew. The results, expressed as  $Q_{O_2}$ , (defined as the oxygen consumption in mm.<sup>3</sup>/hr./mg. dry weight), were as follows:

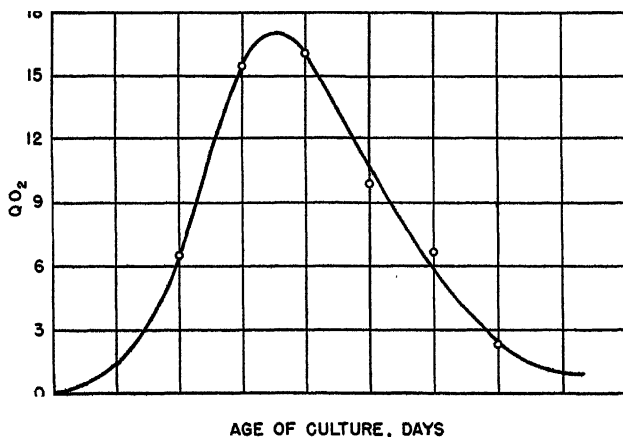


FIG. 1. The  $Q_{O_2}$  of *Penicillium notatum* in Cultures of Different Ages, as Measured in a Lactose—Corn Steep Medium.



Thus, if the oxygen consumption values for cultures of different ages are compared in relation to the dry weights of mycelia produced, it is apparent that the oxygen consumption of *P. notatum* is a function of the age of the mycelium, rising to a maximum at 3–4 days and decreasing rapidly thereafter. When the increments of growth of the cultures are also taken into account (Table I), it may be observed that the oxygen consumption of the entire culture becomes maximal slightly later, at 4–5 days. An approximate calculation places the oxygen consumption per culture, under the conditions here specified, at close to 1 l. during the growth period of 7 days.

TABLE I

*The Relation of Age of Culture,  $Q_{O_2}$ , and Dry Weight of Mycelia of *P. notatum*, Grown in Corn Steep Media, and Whose Respiration was Measured in the Media in Which They Were Grown*

Age	$Q_{O_2}$	Dry wt. of mycelium,	$Q_{O_2} \times \text{dry wt.}$
<i>days</i>		<i>mg.</i>	
2	6.33	—	—
3	15.39	352	6455
4	16.20	660	10692
5	10.03	957	9570
6	6.53	1083	7039
7	2.21	1228	2720

It has been noted that the  $Q_{O_2}$  of this strain of *P. notatum*, when grown for 6 days in the corn steep medium, was 6.53 according to the determination presented in Table I. When the mycelia were removed from the corn steep medium at this time, and placed in distilled water for 20–24 hours, depleting the food reserves, the  $Q_{O_2}$ , as measured in non-nutrient buffer, was decreased to a lower value, subject to considerable variation among the different experiments, which averaged  $1.67 \pm 0.51$  (based upon 50 experiments). Experiments with other fungi (9, 11), have demonstrated the necessity of such a starvation procedure. In mycelia removed from the culture media and used immediately in respirometer experiments, little if any increase in respiratory rate over that of the control could be obtained upon the addition of a respirable substrate, indicating the presence within the mycelia of food reserves sufficient to enable metabolism to proceed at a maximal rate.

The effects of various carbohydrates, supplied in *M/10* concentration, upon the oxygen consumption of *P. notatum* are shown in Table II. Results of two separate determinations are presented in most instances. The results are expressed in both absolute and relative terms; *i.e.*, as  $Q_{O_2}$  in non-nutrient buffer, as  $Q_{O_2}$  of identical mycelia in the presence of the given carbohydrate, and also, in order to facilitate comparisons between different substrates, as percentage increase in oxygen consumption. The latter figures are in most instances averages, to the closest 5%.

Negative results, or weakly positive results of dubious significance, were obtained in similar experiments with mannitol, sorbitol, dulcitol and adonitol. To determine whether or not these substances were oxidized by *P. notatum*, the starvation period was increased to 48 hours, and the experiments were then repeated, with the results shown in Table III.

In an attempt to secure some insight into the mechanisms of cellular respiration in *P. notatum*, the respiratory poison potassium cyanide was employed. With the mycelia suspended in buffer in the presence of a respirable substrate, namely *M/10* glucose, a quantity of cyanide known to have a maximal effect upon the respiration of other fungi (10) was introduced, and the respiratory rate was compared with that found in the absence of cyanide. In two such experiments using *M/100* KCN, the decrease in oxygen consumption in the presence of cyanide was approximately 65% in each instance.

## DISCUSSION

The data on oxygen consumption of this surface strain of *P. notatum* in a corn steep medium cannot be directly compared with the findings of Köffler *et al.* (6), who made similar studies on submerged strains. These workers reported their results as  $Q_{O_2}$  (ml.); that is, the oxygen consumption of the culture solution plus the mycelium it contained, the amount of mycelium not being reported. Variation in the oxygen consumption of the mycelium with age of the culture, as noted herein, was also observed in submerged strains: "After the  $Q_{O_2}$  (ml.) values had passed their maximum, their decline is not only due to smaller amounts of active protoplasm present, but is also due to decreased respiratory activity per unit of protoplasm" (6). Because of the small amount of oxygen required by the surface strain of *P. notatum*, it is

TABLE II

*The Action of Various Carbohydrates on the Oxygen Consumption of P. notatum*

Carbohydrate	Original $Q_{O_2}$	Final $Q_{O_2}$	Percentage increase in oxygen consumption
Corn steep solids (1.8%)*	1.88	3.63	95
Glycerol	1.00 1.52	1.31 2.25	40
Calcium lactate	2.66	4.18	55
d-Arabinose	1.68	2.03	20
d-Xylose	1.51 1.85	1.87 2.52	30
Rhamnose	1.70	2.18	30
Glucose	1.20 1.80	3.18 3.53	130
Levulose	2.17 2.47	3.06 3.41	40
Galactose	1.49 0.93	2.91 2.21	115
Mannose	3.07 2.50	5.74 4.32	80
Sucrose	2.68 1.10	3.43 1.75	45
Lactose	1.74 2.11	2.26 2.76	30
Maltose	1.37 1.14	2.74 2.60	115
Cellobiose	1.27 1.74	2.49 3.88	110
Trehalose	2.65	2.70	0
Dextrin (1.62%)*	2.16 0.94	3.33 1.57	60
Soluble starch (1.62%)*	1.51 2.65	1.40 2.54	0

\* In these materials of unknown composition, the concentrations used were as stated.

TABLE III

*The Action of Various Sugar Alcohols upon the  
Oxygen Consumption of P. notatum*

Carbohydrate	Original Q <sub>O<sub>2</sub></sub>	Final Q <sub>O<sub>2</sub></sub>	Percentage increase in oxygen consumption
Mannitol	0.96	1.45	50
Sorbitol	1.12	1.89	70
Dulcitol	1.11	1.33	20
Adonitol	0.86	1.30	50

probable that lack of oxygen is never a limiting factor in the production of penicillin by the surface process.

Since commercial penicillin production employs a medium in which the carbohydrates present are corn steep solids, glucose and lactose, the relative rates of oxidation of these and related compounds are of some interest. According to Moyer and Coghill (7a), the principal carbohydrate constituents of corn steep solids are glucose, the dextrans, and lactic acid and its salts, the proportion of the latter with respect to glucose varying with the degree of fermentation which the steep liquor has undergone prior to its incorporation into culture media. These workers have found that the content of assimilable compounds in corn steep solids is alone sufficient to support fairly good fungus growth and moderate penicillin production in the absence of added carbon sources.

As might be expected of a member of this cosmopolitan genus of fungi, *P. notatum* possesses a large number of carbohydrases, enabling it to make use of a wide variety of carbohydrates. The present data indicate that glucose, galactose, mannose, maltose and cellobiose are rapidly oxidized by *P. notatum*. Other carbon sources, which are more slowly oxidized, include glycerol, calcium lactate, arabinose, xylose, rhamnose, levulose, sucrose, lactose, dextrin, mannitol, sorbitol, dulcitol and adonitol. Trehalose and soluble starch appear not to be oxidized by this strain of *P. notatum*, under these experimental conditions.

Evidence showing that starch is an adequate carbon source for *P. notatum* in surface cultures has been presented by Moyer and Coghill (7a). A probable explanation of the failure of the fungus to oxidize soluble starch in the present experiments lies in the fact that, prior to

oxidation, the starch must first be hydrolyzed to reducing sugars, a process presumably much slower than the subsequent oxidation. Hence, the period during which the mycelium and soluble starch were in contact in our experiments was probably insufficient to permit the formation of an amount of sugar sufficient to give rise to an increased oxygen consumption.

In the study of Koffler *et al.* (6) upon submerged strains of *P. notatum*, data obtained with the Warburg respirometer indicated that glucose is oxidized more rapidly than sucrose, and sucrose more rapidly than lactose. The present data involving a surface strain are in agreement. Moyer and Coghill (7a), by sugar analyses made on surface cultures, also found that glucose is consumed more rapidly than is lactose.

As a sole source of carbon, lactose supports only a trace of growth by *P. notatum* (7a, 7b). Moyer and Coghill are of the opinion that the superiority of lactose over other carbohydrates in producing a high yield of penicillin is associated with a slow rate of assimilation of this compound by the fungus. The present findings as to the relatively slow rate of oxidation of lactose by *P. notatum* would also appear to support the idea that the great value of lactose in commercial penicillin production is causally related to the fact that lactose is a relatively poor carbon source, which is oxidized at a slow and constant rate throughout the period of growth and activity of the mold.

It appears that certain of the hexoses, and disaccharides which yield only glucose upon hydrolysis, are the best carbon sources for *P. notatum*. Pentoses are very poor sources of carbon for this organism, being oxidized very slowly.

The observation that glycerol is oxidized by *P. notatum* is supported by Moyer and Coghill's (7a) finding that this substance is apparently utilized in growth of the fungus. The same is true of sorbitol (7a), and presumably of the related compounds mannitol, dulcitol and adonitol.

Evidence for the utilization of lactate, an important ingredient of corn steep liquor, is provided in Moyer and Coghill's (7a) experiments in which stimulation of growth was noted upon addition of lactic acid to media low in glucose and low in corn steep solids. That lactate is actually oxidized is proved by the present experiments.

The present data from respirometer experiments agree completely with previous findings concerning the utilization of carbohydrates in

both surface and submerged penicillin production (6, 7a, 7b). There appears to be no necessary correlation, however, between the rates of oxidation of various compounds (and their utilization in growth and metabolism of *P. notatum*) and the production of penicillin.

Additional experiments with potassium cyanide, performed over a wide range of cyanide concentrations, and possibly experiments with other specific inhibitors of cellular respiration, would be required to obtain information as to the mechanisms of cellular oxidations in *P. notatum*. In the present experiments, no special precautions were taken to prevent distillation of cyanide into the alkali, a factor of considerable importance in certain instances. Examination of the present experiments in the light of the data of Robbie, Boell and Bodine (8) on this point reveals, however, that this factor could not have accounted for an error of more than 5% in the cyanide effect. Since the observed decrease in respiratory rate in the presence of *M*/100 KCN was 65%, it would appear that a considerable amount of the oxidative metabolism in *P. notatum* is carried out by a "cyanide-sensitive" respiratory system, involving cytochrome.

#### ACKNOWLEDGMENTS

The writer's sincere thanks are expressed to Mr. H. A. Kaufmann of the American Maize-Products Company, who provided the corn steep solids used in these experiments, to Prof. C. S. Shoup and Prof. W. M. Deacon of this Department for certain of the chemicals employed, and to Prof. Shoup for advice and assistance in the respirometer experiments.

#### SUMMARY

This study is concerned with measurements of the oxygen consumption of a surface strain of *Penicillium notatum* (NRRL 1249), using the Fenn differential respirometer.

The  $Q_{O_2}$  of *P. notatum*, as measured in the lactose-corn steep medium in which the fungus was grown, varies with the age of the culture, increasing to a value above 16 mm.<sup>3</sup>/hr./mg. at 3–4 days, and decreasing rapidly thereafter.

Glucose, galactose, mannose, maltose and cellobiose are rapidly oxidized by *P. notatum*. Glycerol, calcium lactate, arabinose, xylose, rhamnose, levulose, sucrose, lactose, dextrin, mannitol, sorbitol, dulcitol and adonitol are more slowly oxidized. Trehalose and soluble starch were not oxidized, under the conditions employed, by this strain of *P. notatum*.

The significance of these findings in relation to commercial penicillin production is discussed.

A portion of the cellular respiration of *P. notatum* is carried out by a "cyanide-sensitive" system, involving cytochrome.

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# The Apparent Concentration of Free Tryptophan, Histidine and Cystine in Normal Human Urine Measured Microbiologically\*

Willi Frankl and Max S. Dunn

*From the Chemical Laboratory, University of California, Los Angeles*

Received September 23, 1946

## INTRODUCTION

It has long been evident that accurate data on the urinary excretion of free and combined amino acids under normal and pathological conditions would aid greatly in the understanding of amino acid metabolism, and probably would be of clinical significance. Although values have been reported for almost all of the amino acids, some of the data may lack quantitative significance owing to the presence of interfering substances in urine, the low concentration of some amino acids in urine and the non-specificity of some analytical procedures.

The adaptation of microbiological procedures to the determination of amino acids in urine was undertaken because of the convenience of such methods and the relatively high sensitivity of the microorganisms commonly employed for assay purposes. The values given in the present paper for apparent free cystine, histidine and tryptophan in urine necessarily include any other substances with equivalent activity for the stated microorganisms. Hegsted (2) has reported that the keto and hydroxy analogues of leucine, isoleucine and valine were active toward

\* Paper 34. For Paper 33 see Shankman *et al.* (1). This work was aided by grants from the John and Mary R. Markle Foundation, the Nutrition Foundation, Inc., and the University of California. The Army K and Army C Rations were furnished by the Military Planning Division, Research and Development Branch, Office of the Quartermaster General, Army Service Forces, for use by one of us (M.S.D.) on Project No. R 49001-QM-382.

The authors are indebted to M. N. Camien, S. Shankman, H. F. Schott and L. B. Rockland for valuable suggestions and technical assistance. They acknowledge with thanks the technical aid rendered by the 20 male and female subjects.



*Lactobacillus arabinosus* although the activity of these substances usually was less than that of the corresponding amino acids. Acetyl-leucine and glycylleucine were found to be active while the acetyl and benzoyl derivatives of isoleucine and valine were inactive. Whether or not *L. arabinosus* and *L. mesenteroides* would respond similarly to the corresponding analogues and derivatives of cystine, histidine and tryptophan has not been determined. It appears probable, however, that the apparent concentrations found in the present experiments may represent primarily free amino acids since there was no evidence of drift in the assays due to inhibition or stimulation, the values calculated for the different levels of sample were in reasonably good agreement, untreated and ether-extracted urines contained approximately the same apparent free-tryptophan content, and the observed urinary concentrations generally were lower than those obtained by chemical methods.

### EXPERIMENTAL

Eleven male and nine female subjects were maintained on uncontrolled normal diets and six of the males subsisted for separate five-day periods on Army C and Army K Rations. The water intake was uniform at all times during the experiments on Army Rations but, on the normal diets, it was regulated only from dinner until the time of collection of the first fasting sample. The latter were collected about 13 hours after dinner. The urine samples were preserved by the addition of toluene and by storage in a refrigerator. The observation that tryptophan is not destroyed under these conditions is in agreement with the recent report of Schweigert *et al.* (3). It is of interest that Albanese and Frankston (4) have found that tryptophan decomposes rapidly in urine which has been strongly acidified with HCl and preserved with 10% alcoholic thymol.

Tryptophan was determined in urine samples, diluted about 1:40, with *Lactobacillus arabinosus* 17-5 essentially by the microbiological procedure of Greeno and Black (5). Histidine was determined in urine samples, diluted about 1:160, with *Leuconostoc mesenteroides* P-60 and the basal medium given as Medium D in Table I of a previous paper (6) except that 1.3 times the stipulated concentrations of total amino acids were employed. Cystine was determined in urine samples, diluted about 1:40, with *L. mesenteroides* P-60 and the basal medium given as Medium D (concentrations given  $\times 1.3$ ) in Table I of a previous paper (6). The final volume of solution in each tube was 3 ml. The techniques used were those described in previous publications from this laboratory.

The probable accuracy of the assay data is indicated by the following mean deviations from the mean for the values at the different levels of samples: *Tryptophan*. Range, 1.5-10%. Most assays, 2-3%. *Histidine*. Range, 1.4-8%. Most assays, 2-3%. *Cystine*. Range, 3.4-10% for all assays except 8 which were 11-16%.

TABLE I  
*Excretion of Tryptophan, Histidine and Cystine on K Ration*

Male† subject	Tryptophan				Histidine				Cystine			
	Output/hour				Output/hour				Output/hour			
	Fasting experiment§		22-24 hours on diet		Fasting experiment		22-24 hours on diet		Fasting experiment		22-24 hours on diet	
	1	2	range**	av.	1	2	range**	av.	1	2	range**	av.
No.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
3	1.67	1.55	1.51-2.46	1.88	6.72	5.42	5.42-12.2	8.76	3.17	2.60	2.60-4.90	3.87
4	1.02	0.87	0.87-1.94	1.27	5.43	4.88	4.88-11.5	7.49	2.89	3.00	2.89-5.80	4.01
5	0.46	1.64	0.46-1.77	1.31	27.8	1.81*	1.81-10.3	7.65	1.10	4.40	1.10-4.40	3.14
7	1.38	1.24	1.24-2.44	1.69	41.0	6.80	4.44-11.9	7.40	2.42	1.87	1.87-4.23	2.93
8	1.10	1.24	1.05-2.90	1.59	34.2	4.36	4.51-15.2	9.57	3.17	3.26	3.26-5.80	4.29
9	1.29	1.63	1.29-2.36	1.82	44.4	6.60	6.60-14.3	9.77	2.60	3.63	2.60-5.87	4.57
Average	1.15	1.36	0.46-2.90†	1.59	37.0	5.98	4.44-15.2†	8.44	2.56	3.13	1.10-5.87†	3.80

\* Omitted in calculating the average value.

\*\* The number of samples were 6 in one case, 7 in four cases, and 10 in one case. The maximum rate of excretion, represented in each case by the largest value, occurred 3-4 hours following the ingestion of food.

† The ages in years and weights in pounds, respectively, given in the parentheses were: No. 3 (24, 165), No. 4 (24, 160), No. 5 (23, 125), No. 7 (33, 145), No. 8 (26, 169) and No. 9 (40, 151).

‡ Maximum range.

§ Three-hour fasting samples were collected about 13 hours after dinner on the fourth and fifth days of the five-day experimental period in the experiments on K and C Rations. (Table II).

## DISCUSSION

It was observed (Table I) that the rate of excretion of tryptophan, histidine and cystine was maximum about 3-4 hours following the ingestion of food. These findings are in harmony with those of Folin and Berglund (7) who reported in 1922 that the hourly excretion of  $\alpha$ -amino acid nitrogen by 6 individuals increased from the average fasting level, 7.3 (4.9-9.6) mg., to as much as 26 mg. within 5-7 hours after the ingestion of glycine (25 g.), casein (60 g.), predigested casein (10-12 g. of nitrogen) and a normal lunch. The level of excretion was considerably higher after 135 g. of gelatin were eaten. Folin and Berglund concluded that there was no threshold concentration of  $\alpha$ -amino acid nitrogen and there appeared to be none for the amino acids studied in the present experiments. Although it was suggested by Folin (8) that normal and pathological metabolism might be measured satisfactorily from the levels of urinary constituents determined in 3- or 4-hour fasting urines, this procedure has not been found practicable in the present work because of the marked differences in urine output in pathological cases.

*Tryptophan.*<sup>1</sup> It may be noted from the data given in Tables I-III for male subjects that the average 24-hour output of tryptophan was 37.0 (27.8-44.4) mg. on K Ration, 19.9 (15.1-26.7) mg. on C Ration and 18.0 (12.8-22.9) mg. on normal diets. The last two figures are in good agreement with the average value, 20.5 (12.6-30.5) mg. of tryptophan, reported recently by Schweigert *et al.* (3) but they are less than 0.1 the average value, 282 (226-350) mg., found earlier by Albanese and Frankston (4). Schweigert *et al.* pointed out that any *d*(+)-tryptophan, or any tryptophan or indole derivatives in combination with other molecules, might be measured by chemical methods but probably would exhibit little, or no, activity toward *L. arabinosus*. On the other hand, both Schweigert *et al.* and Albanese and Frankston found that the apparent tryptophan content of urine extracted with

<sup>1</sup> It has been reported recently by Schweigert *et al.* (9) that "mouse urine extracted with ether yielded essentially the same values for tryptophan as did unextracted urine." Tryptophan was determined by microbiological assay with *L. arabinosus*. It was found that the daily urinary excretion of apparent tryptophan by a squad of college women, who subsisted on diets supplying 30 g. of protein daily as either whole eggs or soy beans, averaged 6.9 (5.5-9.8) mg. About 1.5% of the estimated tryptophan ingested was accounted for by the urinary tryptophan.

TABLE II  
*Excretion of Tryptophan, Histidine and Cystine on C Ration*

Male subject	Tryptophan			Histidine			Cystine		
	Fasting output/hour experiment		24-hour output	Fasting output/hour experiment		24-hour output	Fasting output/hour experiment		24-hour output
	1	2		1	2		1	2	
no.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
3	1.12	1.10	26.7	9.45	7.45	288	4.40	4.36	117
4	0.32	0.54	15.6	3.22	7.62	231	1.90	3.71	126
5	0.64	0.93	15.1	7.15	11.6	207	3.42	5.75	81
7	0.80	0.33	26.2	6.62	3.29	306	3.32	1.18	112
8	0.64	0.72	16.0	4.62	7.65	226	3.33	4.22	113
9	0.68	0.82	19.5	6.47	7.45	232	4.25	4.76	132
Average	0.70	0.74	19.9	6.25	7.51	248	3.44	4.00	114

TABLE III  
*Excretion of Tryptophan, Histidine and Cystine on Normal Diet*

Male* subject	Tryptophan			Histidine			Cystine		
	Fasting output/hour experiment		24-hour output	Fasting output/hour experiment		24-hour output	Fasting output/hour experiment		24-hour output
	1	2		1	2		1	2	
no.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
1	0.71	0.66	22.9	4.00	3.63	177	2.82	2.62	107
2	1.0	0.94	21.1	9.75	7.90	201	6.20	7.10	166
3	0.76	0.77	18.6	8.05	6.46	202	2.90	2.30	77
5	0.60	0.92	13.3	5.80	15.3	191	2.58	4.16	55
6	0.54	0.65	12.8	4.95	7.45	114	4.37	5.47	126
7	0.77	0.91		7.05	7.40		2.59	3.93	
8	0.51	0.54	15.2	4.29	4.87	180	2.80	3.12	93
9	0.69	0.69	19.0	5.45	6.81	202	3.06	3.39	104
10	0.73	0.71	22.9	4.10	4.60	98	4.15	4.20	132
11	0.56	0.72	15.8	6.12	8.55	206	3.45	4.23	110
Average	0.69	0.75	18.0	5.96	7.30	175	3.49	4.05	108

\* The ages in years and weights in pounds, respectively, given in the parentheses were: No. 1 (19, 150); No. 2 (24, 215)—vegetarian; No. 6 (25, 135); No. 10 (29, 150); and No. 11 (34, 150). The data for the other subjects are given in a footnote to Table I.

ether was the same as, or only slightly less than, that of untreated urine. The same observation has been made in the present experiments. Albanese and Frankston showed, in addition, that neither tryptamine nor acetyl-*dl*-tryptophan gave a direct Jolles color reaction and that the color-producing substances (indole, indoleacetic acid and indolepropionic acid) tested were removed from urine by a single ether extraction. Of interest in this connection is the report of Borchers *et al.* (10) that kynurenic acid and kynurenine in urine may account for one-third to one-half of the tryptophan fed to experimental animals.

TABLE IV  
*Excretion of Tryptophan, Histidine and Cystine on Normal Diet*

Female subject*	Tryptophan 24-hour output	Histidine 24-hour outp	Cystine 24-hour output
No.	mg.	mg.	mg.
1	6.9	57	36
2	15.9	149	48
3	15.7	133	29
4	9	118	184
5	21.6	92	158
6	15.4	128	115
7	11.7	74	85
8	19.3	173	113
9	11.9	130	137
Average	14.1	117	100

\* The ages in years and weights in pounds, respectively, given in the parentheses were: No. 1 (22, 165), No. 2 (48, 186), No. 3 (36, 118), No. 4 (35, 112), No. 5 (65, 145), No. 6 (33, 122), No. 7 (35, 120), No. 8 (34, 110), and No. 9 (32, 92).

It was found by the present authors that an untreated 24-hour urine containing approximately 270 mg. of apparent tryptophan, according to an analysis by the Albanese-Frankston method, contained no apparent tryptophan as determined either by the Albanese-Frankston chemical method or by microbiological assay after it had been treated with chloramine-T essentially as described by Van Slyke *et al.* (11). According to Van Slyke *et al.*,  $\alpha$ -amino acids are nearly completely destroyed by chloramine-T although the non-tryptophan peptides investigated were only slightly attacked. It appears possible, however,

that any tryptophan-containing peptides present in urine would be destroyed by chloramine-T.<sup>2</sup>

It was proposed by Albanese and Frankston that urinary tryptophan may be derived from normal tissues rather than from dietary materials but the correlation found between tryptophan excretion and body weight was not observed either by Schweigert *et al.* or the present investigators. It is considered probable that tryptophan excretion may be related to one or more factors in addition to body weight. Holt *et al.* (12) have estimated that 6-9 mg. of *l*(-)-tryptophan/kg. are required daily to maintain an adult in nitrogen equilibrium and to maintain the urinary excretion of this amino acid at the normal level. Even though substances other than free tryptophan may have been measured, the colorimetric tryptophan data of these investigators may provide an adequate basis for these conclusions.

There is no apparent explanation, other than possible differences in amino acid composition of the diets, for the observation (Tables I and II) that the 24-hour output of apparent tryptophan on K Ration was nearly 100% higher than that on C Ration and normal diets. It is of interest that the average 24-hour excretion of tryptophan by the female subjects was 14.1 (9-21.6) mg. and that this value is approximately 25% lower than that found for male subjects on normal diets.<sup>1</sup>

*Histidine.* The average 24-hour output of apparent histidine by the male subjects was 196 (148-236) mg. on K Ration, 248 (207-306) mg. on C Ration and 175 (98-206) mg. on the normal diets. The average 24-hour output by the female subjects was 117 (57-173) mg.

The presence of histidine in urine was first established in 1908 by Engeland (13) who isolated this amino acid as its picrolonate. The isolation of 0.2 g. of nearly pure histidine monohydrochloride from 10 l. of human pregnancy urine was reported by Armstrong and Walker (14) in 1932. Ten years later, Edlbacher and von Bidder (15) isolated 11 g. of purified histidine monohydrochloride from 40 l. of human pregnancy

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<sup>2</sup> Dr. A. A. Albanese has stated in a personal communication dated July 18, 1945, that "A number of unreported experiments have shown that our colorimetric technique yields a red color with tryptophan in peptide as well as in other linkages. Furthermore, unreported data from our human studies suggest that a part of the urinary tryptophan escapes reutilization because it occurs in a bound form. It is conceivable, therefore, that the discrepancy between the chemical and the biological methods which you and Elvehjem (refers to Schweigert *et al.* (3)—WF and MSD) note arises from the fact that the microorganism, like the human, is unable to utilize the bound tryptophan, so that your values represent only free tryptophan, whereas our values represent total (free plus bound) tryptophan. We suspect that a similar condition exists with respect to urinary arginine. And as you know, Dr. Sullivan has long reported the occurrence of this relationship with regard to cystine." The writers are indebted to Dr. Albanese for permission to publish this statement.

urine. Histidine has been determined in urine primarily by the Pauly diazotized sulfanilic acid and the Knoop bromine colorimetric methods. The daily urinary excretion of histidine by normal adults has been estimated to be 150–600 mg. (16), 150 mg. (14), 100–300 mg. (17), 20–200 mg. (18), 0–400 mg. (19), 90–1025 mg.<sup>3</sup> (20) and 180–760 mg. (21) by the authors cited. The recently estimated value, 15–50 mg. % (22), would be equivalent to 150–500 mg. calculated for a 24-hour volume of 1500 ml. Albanese *et al.* (23) have reported that the normal histidine excretion by adult males was unchanged on a histidine-deficient diet although an abnormal metabolite which gave a green color reaction with the Sharlit indican reagent appeared in the urine.

*Cystine.* The average 24-hour output of apparent cystine by the male subjects was 90 (71–115) mg. on K Ration, 114 (81–132) mg. on C Ration and 106 (55–166) mg. on the normal diets. The average 24-hour output by the female subjects was 100 (36–184) mg. The daily excretion by normal adults has been reported by the authors cited to be 0–180<sup>4</sup> mg. (24), 65–113<sup>5</sup> mg. (25), 16.5–57<sup>6</sup> mg. (26), 41–95 mg. and 81–159 mg. for fresh urine, and 69–148 mg. for acid-hydrolyzed urine<sup>7</sup> (27), 15–70<sup>8</sup> mg. (28), 42–80 mg. (29), and 40–80<sup>9</sup> mg. of free cystine and 100–200<sup>10</sup> mg. of total cystine (30).

<sup>3</sup> Estimated for 1500 ml. volumes from the values, 6–75 mg., found/100 ml. of urine.

<sup>4</sup> Estimated for 1500 ml. volumes from the values, 0–12.0 mg., found/100 ml. of urine. Cystine was determined by Looney's (31) colorimetric method and nearly theoretical amounts of cystine added to normal urine were recovered.

<sup>5</sup> Estimated for 1500 ml. volumes from the values, 1.1–7.5 mg. found/100 ml. of urine. The higher values, 11.8 and 12.5 mg./100 ml. of urine, were found in two urines of supposedly normal individuals. Cystine was determined by a modification of the Okuda iodometric titration procedure, and an average of 101 (96–105)% of cystine added to normal human and rabbit urines and to human cystinuric urines was recovered.

<sup>6</sup> Estimated for 1500 ml. volumes from the values, 1.1–3.8 mg. found/100 ml. of urine. The values, 0.0–0.9 mg. of cysteine/100 ml. of urine were also reported. The authors' phospho-18-tungstic acid methods for the differential determination of cystine and cysteine were employed. It was stated that good recovery was obtained of cystine and cysteine added to oxidized urine.

<sup>7</sup> The values, 81–159 mg., were obtained by a modified Okuda iodometric titration procedure. The other cystine values were determined by the authors' NaCN-naphthoquinone sulfonate colorimetric method. The recoveries of cystine added to urine ranged from 73–98%.

<sup>8</sup> Estimated for 1500 ml. volumes from the values, 0.84–4.68 mg., found/100 ml. of urine. Cystine was determined by reaction with cuprous chloride and colorimetric determination with phospho-18-tungstic acid of the cysteine in the precipitated copper mercaptide according to the procedure of Medes and Padis (32). The values found by other methods were 14–117 (Shinohara), 22–152 (Virtue and Lewis), 20–25

## SUMMARY

The apparent free tryptophan, histidine and cystine in normal urine has been determined by microbiological procedures. It has been found that the hourly output of these amino acids increased after meals and that the fasting rate of excretion was variable. The approximate average daily urinary excretion of these amino acids by male subjects was found to be 20 mg. of tryptophan on C Ration and normal diets, 40 mg. of tryptophan on K Ration, 250 mg. of histidine on C Ration, 200 mg. of histidine on K Ration, 175 mg. of histidine on normal diets and about 100 mg. of cystine on K Ration, C Ration and normal diets. The comparable average values for female subjects on normal diets were about 25% lower for tryptophan, about 35% lower for histidine and about the same for cystine. The values for these three amino acids found in the present experiments by microbiological methods were approximately of the same order of magnitude as those obtained by some other workers using colorimetric methods.

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<sup>9</sup> Cystine was determined by the Shinohara method and cystine added to urine was quantitatively recovered.

<sup>10</sup> Cystine was determined by a polarographic method. Essentially the same results were found by the colorimetric procedure of Sullivan and Hess (27).



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# The Apparent Concentration of Free Tryptophan, Histidine and Cystine in Pathological Human Urine Measured Microbiologically\*

Willi Frankl, Helen Martin and Max S. Dunn

*From the Chemical Laboratory, University of California, Los Angeles,  
and the Department of Medicine, University  
of Southern California, Los Angeles*

Received September 23, 1946

## INTRODUCTION

The daily excretion of apparent free tryptophan, histidine and cystine in the urine of normal male and female subjects, determined by microbiological procedures, was reported in the preceding paper(1). The urinary excretion of these amino acids by 57 pathological individuals has been investigated in the present studies. The types of disorders included 15 cases of liver disease, 9 of diabetes mellitus, 8 of malignancy, 7 of kidney disease, 6 of pregnancy, 4 of blood diseases, 4 of acute infections, 2 of bronchial asthma, 1 of toxic goitre and 1 of dermatomyositis.

## EXPERIMENTAL

The urine samples were preserved by adding toluene and storing them in a refrigerator. The amino acids were determined by the microbiological procedures described previously (1). The values were considered to be satisfactory since the mean deviations from the mean for the values at the different levels of samples were approximately the same as those obtained previously with normal human urine (1).

## DISCUSSION

It may be observed from the data given in Tables I and II that about one-half (56%) of the 157 amino acid values were within the ranges considered to be normal for male and female subjects. Although

\* Paper 35. For Paper 34 see Frankl and Dunn (1). This work was aided by grants from the John and Mary R. Markle Foundation, the Nutrition Foundation, Inc., and the University of California.

TABLE I  
Daily Urinary Excretion of Apparent Free Tryptophan, Histidine and Cystine by 57 Pathological Individuals\*

Subject			Diagnosis	Urine volume (24 hr.)	Tryptophan	Histidine	Cystine	Treatment
Number	Race	Sex						
Liver Disease <sup>a</sup>				ml.	mg.	mg.	mg.	
1	W	F	Late subacute portal cirrhosis; pulmonary tuberculosis (B)	1680	13	204	245	Choline chloride, 6 g./24 hrs.
2	W	M	Probable subacute portal cirrhosis (C)	1340	19.1	40	76.6	Choline chloride, 3 g./24 hrs. crude liver extract
3	W	M	Subacute portal cirrhosis with necrosis (C)	2640	42	234	172	Choline chloride, 6 g./24 hrs. liver extract
4	W	M	Subacute cirrhosis (C)	390	18.1	38.8	141	Choline chloride, 2 g./24 hrs.
5	W	M	Subacute portal cirrhosis with ascites (C)	2500	3.1	18.8		Choline chloride, 6 g./24 hrs. vitamins
6	W	F	Subacute portal cirrhosis with ascites (C)	610	16.4	139	83	Choline chloride, 6 g./24 hrs. liver extract
7	W	M	Probable early subacute portal cirrhosis; nutritional deficiency (C)	3670	18.4	67.5	117	Vitamins
8	W	M	Probable subacute cirrhosis; chronic alcoholism; arteriosclerotic heart disease in failure (C)	543	11.4	23	51.5	Vitamins; digitalis
9	W	F	Chronic portal cirrhosis with necrosis (C)	3200	94	61.2	13	Vitamins; liver extract
10	W	M	Chronic portal cirrhosis with acute necrosis; generalized peritonitis (A)	5175	26.5	1183	392	Probably 2 g. choline chloride only; vitamins

\* The notations are as follows: A, autopsy; B, biopsy; C, clinical and laboratory; F, female; J, Japanese; M, male; N, Negro; W, White; Ch, Chinese.

<sup>a</sup> See, also, subjects number 16 and 17.

TABLE I (Continued).  
Daily Urinary Excretion of Apparent Free Tryptophan, Histidine and Cystine by 57 Pathological Individuals

Subject				Diagnosis	Urine volume (24 hr.)	Tryptophan	Histidine	Cystine	Treatment
Number	Race	Sex	Age						
Liver Disease (Cont.) yrs.									
11	W	M	45	Chronic portal cirrhosis with bleeding varices (C)	750	30.2	111	45	Vitamins; liver extract; transfusions
12	J	M	57	Chronic portal cirrhosis with ascites (C)	560	10.6	22		Vitamins; paracentesis
13	N	M	21	Arsenical hepatitis (C)	820	12.8	51	214	Choline chloride, 6 g./24 hrs. liver extract; vitamins
14	W	F	54	Infectious hepatitis (C)	2315	17.6	129	79	Choline chloride, 3 g./24 hrs.
15	W	F	46	Hypertensive heart disease with failure and cardiac cirrhosis (C)	1220	9.5	41.7	38.2	Digitalis
Diabetes Mellitus									
16	W	M	37	Controlled diabetes mellitus; hepatomegaly (? type) (C)	2650	31.8	134		70 units insulin/24 hrs.
17	W	F	48	Controlled diabetes; hepatitis; chronic intermittent obstruction	2275	12.2	54.5		20 units insulin/24 hrs; choline chloride, 1.5 g./24 hrs.
18	W	M	55	Controlled diabetes; intercapillary glomerulosclerosis in azotemia with salt retention (C)	1150	16.1	55		No insulin; low salt diet
19	W	M	49	Probable diabetes; intercapillary glomerulosclerosis in azotemia (A)	1220	1.8	21.4	250	Digitalis
20	W	M	21	Controlled diabetes (C)	2880	17.7	76.4		80 units insulin/24 hrs.
21	W	M	36	Controlled diabetes; infected foot; chronic diarrhea (C)	3220	42.2	62.5		90 units insulin/24 hrs.

TABLE I (Continued)  
Daily Urinary Excretion of Apparent Free Tryptophan, Histidine and Cystine by 57 Pathological Individuals

Subject			Diagnosis	Urine volume (24 hr.)	Tryptophan	Histidine	Cystine	Treatment
Number	Race	Sex						
Diabetes Mellitus (Cont.)								
22	W	M	19	2440	22.	88.6	124	65 units insulin/24 hrs.
23	W	M	25	2160	14.9	83.4	76	70 units insulin/24 hrs.
24	W	F	32	5250	58.5	217	187	Insulin and intravenous fluids
25	Ch	M	40	2175	22.6	136		630 units insulin/24 hrs.
26	J	M	43	4680	29.2	164	119	30 units insulin/24 hrs.
27	W	M	67	2100	2.8	16.6		No insulin
Kidney Disease <sup>b</sup>								
28	W	M	46	1780	12.6	24.6		
29	W	M	28	470	5.3	6.2	49.2	
30	N	F	49	640	17.4	80.8	77.5	Low salt diet
31	W	F	56	1100	15.4	31	152.5	Choline chloride. g. VI daily

<sup>b</sup> See, also, subjects number 18, 19 and 27.

TABLE I (Continued)  
Daily Urinary Excretion of Apparent Free Tryptophan, Histidine and Cystine by 57 Pathological Individuals

Subject			Diagnosis	Urine volume (24 hr.)	Tryptophan	Histidine	Cystine	Treatment
Number	Race	Sex						
Pregnancy				ml.	mg.	mg.	mg.	
32	N	F	Six months pregnant; pyelitis (C)	1640	18.7	306	108	Sulfathiazole Wheat germ oil
33	W	F	Six months pregnant—bleeding— placenta praevia (C)	2125	27.9	610	93	
34	W	F	Toxemia of pregnancy (8.5 months) (C)	1895	31.6	354	151	
35	W	F	Abortion (complete ?)—2 months (C)	610	2.6	45.9	28	Wheat germ oil Vitamins; intravenous glucose
36	N	F	Three months pregnant; hyperemesis gravidarum (C)	1190	16.6	38	88	
37	W	F	Threatened labor—7 mo., scabies (C)	2800	52	377	154	
Blood Disease								
38	W	M	Pernicious anemia (stage of severe anemia) (C)	1520	25.8	77.5	81.9	Liver extract
39	N	M	Sickle cell anemia with abdominal crisis (C)	2235	16.6	80	108	Transfusions
40	W	F	Monocytic leukemia (C)	2335	7.5	21	89.4	Penicillin
41	W	M	Lymphatic leukemia; lobar pneumonia (B)	1460	19.9	6.25	96.3	
Bronchial Asthma								
42	W	M	Bronchial asthma (C)	3810	12.7	62.5		90
43	W	M	Bronchial asthma (C)	3460	14.4	94.5		

TABLE I (Continued)  
*Daily Urinary Excretion of Apparent Free Tryptophan, Histidine and Cystine by 57 Pathological Individuals*

Subject			Diagnosis	Urine volume (24 hr.)	Tryptophan	Histidine	Cystine	Treatment
Number	Race	Sex						
Acute Infections <sup>a</sup>								
44	N	M	Probable atypical pneumonia with effusion (C)	3480	29.8	85	194	Sulfonamides and penicillin
45	N	M	Lobar pneumonia (C)	3810	14.3	76.2	106	Sulfonamides and aminophylline
46	N	M	Lobar pneumonia (C)	4040	11.2	44	116	Sulfadiazine
47	N	F	Primary atypical pneumonia (C)	1220	6.6	46	70	Aminophylline
Malignancy								
48	W	F	Carcinoma of esophagus (B)	575	5.1	17.3	46	X-ray therapy
49	W	F	Carcinoma of cervix (B)	2020	11	69.7	68	Radium
50	W	M	Carcinoma of hard palate (C)	790	24.8	96		X-ray therapy
51	W	F	Carcinoma of breast (C)	1420	16.2	119	122	Radial mastectomy
52	W	F	Carcinoma of breast with metastases (C)	2550	3.7	38.3	76	X-ray therapy
53	W	F	Reticulum cell sarcoma (jaundice) (C)	770	19.3	55.5	95	X-ray therapy
54	W	F	Carcinoma of breast with metastases (C)	410	13	40.5	79	
55	W	M	Malignant melanoma of leg (B)	3365	8.1	236		Surgical removal of leg
Miscellaneous								
56	W	M	Treated toxic goitre (C)	2000	15.6	145	109	Lugol's solution; thiamine
57	W	F	Dermatomyositis (B)	1020	13.9	74.5		Vitamins; sedation

<sup>a</sup> See, also, subject number 41.





only about 20% of the male tryptophan values and of the male and female cystine values were abnormal, about 15% of the female tryptophan, 60% of the female histidine and 80% of the male histidine values were in this category. It is of particular interest that nearly all of the abnormal male histidine values were below the normal level of urinary excretion. On the other hand, one male histidine value (Subject number 10) and four female histidine (pregnancy) values (Subjects numbers 32-34 and 37) were several-fold larger than those shown previously to be normal. In most cases only one of the three amino acids was excreted at a higher or lower level than the average normal range and an abnormally high rate of excretion of one amino acid was often accompanied by abnormally low excretion of one or two of the other amino acids.

In general, conditions under which low amino acid excretion occurred were those which might influence amino acid synthesis (liver disease), amino acid excretion (kidney disease) and amino acid utilization (malignancy accompanied by malnutrition and anemia). Conditions under which amino acid excretion was high were limited to diabetes mellitus, acute infection, pregnancy and liver disease.

There appears to be no quantitative data in the literature on the nutritionally indispensable amino acid tryptophan. Extensive, although not highly accurate, data are available on the urinary excretion of histidine under pathological conditions. It has been reported that histidinuria is a normal characteristic of human pregnancy (2-16), that histidine excretion is greatly diminished in patients with serious symptoms of preeclamptic toxemia of pregnancy (17-19), that histidine excretion is deranged in various diseased states (20-23) and that the excretion of histidine is partly controlled by the opposing action of histidine decarboxylase and histaminase (24). Studies of cystine in pathological urines have been limited, primarily, to cases of cystinuria. It has been observed that some calculi (25) contain cystine and that cystine excretion may be greatly increased in cystinuria (26-28).

It appears to be a reasonable expectation that data for urinary amino acids may have diagnostic, and possibly therapeutic, significance, particularly in relation to nutritional disorders and diseases of the liver. Investigations of the urinary excretion of these and other amino acids are being continued.

## SUMMARY

The urinary excretion by 57 pathological individuals of tryptophan, histidine and cystine has been determined by microbiological procedures. About one-half of the values were within the ranges considered to be normal for male and female subjects. In general, only one of the three amino acids was excreted at a higher or lower level than the average normal range and an abnormally high rate of excretion of one amino acid was often accompanied by abnormally low excretion of one or two of the other amino acids. The number of abnormal histidine values was 3-4 times that of tryptophan and cystine. Nearly all of the abnormal histidine values were below the normal although, in a few cases, they were several-fold larger than the normal. It was observed that low amino acid excretion occurred primarily in diseases most likely to cause derangement of amino acid synthesis, excretion and utilization. High amino acid excretion was found in some cases of diabetes mellitus, acute infection, pregnancy and liver disease.

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# The Apoenzymatic Nature of Adaptation to Galactose Fermentation\*

S. Spiegelman, John M. Reiner† and Ida Morgan

*From the Department of Bacteriology, School of Medicine, and The Mallinckrodt Institute of Radiology, Washington University, St. Louis, Missouri*

Received December 30, 1946

## INTRODUCTION

*Enzymatic adaptation* is a term applied to the phenomenon observed when cells acquire a new enzymatic activity on incubation in the presence of a specific substrate. Following Karström (1), the enzyme systems which appear under such circumstances have been called *adaptive enzymes* and are distinguished from the *constitutive enzymes*, which supposedly are formed by the cell whether their specific substrates are present in the medium or not. Removal of the substrate from the environment usually leads to the disappearance of the specific adaptive enzyme activity it invoked.

It need hardly be pointed out that, if this phenomenon of induction of enzyme activity by substrate actually represents enzyme formation, it is one of the highest significance. It would permit us to examine the genetic as well as metabolic conditions under which the formation of a functionally well-defined enzyme system is possible. Previous publications (2, 3, 4) have dealt with certain of the genetic and physiological aspects and implications of such phenomena.

Explanations not involving enzyme formation might be proposed to account for the period intervening between the time the cells are first placed in contact with substrate and the appearance of the enzyme activity. For example, the 90–180 minute delay observed before the onset of galactose fermentation could represent the time required for any one or more of the following:

1. The penetration of galactose into the cell.

\* This work was aided by a grant from the American Cancer Society.

† National Research Council Fellow in Zoology, 1946–1947.

2. Raising the concentration of certain of the glucozymase cofactors or intermediates to the level required for the fermentation of galactose.

3. Building up special intermediates (*e.g.*, galactose-1-phosphate) necessary for galactose fermentation but which are foreign to the glucozymase system.

Cases 2 and 3 are analogous to the induction period in the onset of glucose fermentation in cell-free extracts, which is abolished by hexose diphosphate.

There is little doubt that the first mechanism is not involved in galactose adaptation. Galactose actually enters the cell immediately, and is metabolized by a purely aerobic mechanism, before the adaptive fermentative enzymes make their appearance (5). Moreover, whereas yeast juice and maceration extracts prepared from adapted cells are able to ferment galactose, comparable preparations from unadapted cultures are completely inactive (6). Experiments of a similar nature (2), using toluol cytolyzates, led to the same results. In the present paper, analogous experiments with Lebedew preparations are described.

All of these cell-free extract experiments demonstrate that something possessing galactose-fermenting capacity can be extracted from cells after adaptation which was not present before. While they do exclude the involvement of simple penetration as a responsible factor, they do not eliminate the last two possibilities mentioned. Experiments described below, however, make it highly unlikely that either or both are solely or critically involved in the adaptive mechanism.

Consideration of the hypothesis that enzymatic adaptation actually involves the formation of a new enzyme system raises problems of its own. Most enzyme systems contain two components, the so-called *apoenzyme* or protein moiety and the *coenzyme*, which usually consists of one or more diffusible molecules necessary to the activity of the enzyme (7, 8).

The completed system of apoenzyme plus coenzyme is sometimes called the *holoenzyme*. This would include the undivided system of *apozymase* and *cozymase* which is defined by Neuberg and Oppenheimer (9). These authors pointed out that cozymase, now generally known as Coenzyme I, is a well-defined chemical entity, and that numerous similar compounds exist. The term coenzyme refers to the entire class of such enzyme activators, or to any single unspecified member of that class.

Any change in enzyme activity could arise either from a modification of the apoenzyme or of one or more of the coenzymes, or possibly

of both. It is clearly essential to decide which of these changes is involved if the significance of the phenomenon is to be evaluated. It is the major purpose of the present paper to provide the data necessary for such a decision in the case of adaptation to galactose fermentation by yeast.

Despite the relatively extensive experimentation on the phenomenon of enzymatic adaptation, only one attempt to analyze this problem exists in the literature. Euler and Jansson (10) made experiments, using brewer's yeast, which sought to distinguish between these various possibilities in galactose adaptation. In these experiments adapted and unadapted apoenzymes were prepared by washing dried adapted yeast with water, presumably until no fermenting capacity remained with respect to glucose without the addition of coenzyme. As in our experiments, coenzyme was prepared by extraction of whole yeast cells with hot water. It was found that the adapted apoenzyme could be activated to ferment galactose with either adapted or unadapted coenzyme. However, neither kind of coenzyme was capable of conferring galactose-fermenting capacity on the unadapted apoenzyme. These experiments indicate that the modification which occurs during the adaptation takes place in the apoenzymatic component. The fact that little difference was observed whether adapted or unadapted coenzyme was used in the activation of the adapted apoenzyme rules out the possibility that the modification involved both components of the newly-formed enzyme system.

Unfortunately, the decisiveness of these conclusions is weakened by the poor activity of the preparations employed by these authors as well as by the relatively high value of the fermentation in the absence of any added substrate. Thus, *e.g.*, the greatest activity recorded in the presence of substrate yields, on calculation, a  $Q_{CO_2}^N$  of about 0.9, whereas in the complete absence of substrate a value of about 0.4 is attained. The initial high endogenous rate of fermentation makes it difficult during the first 3 hours of the experiment to distinguish between the sample which has added substrate and the one which does not. On the other hand, the data obtained in the last 3 hours are marred by a progressive decline in the activity of the enzyme preparation.

In view of the importance of such experiments and the conclusions derivable from them, both for the understanding and the use of the phenomenon of enzyme adaptation, it seemed desirable to reinvestigate the problem, employing different procedures.

### *Materials and Methods*

The strain of yeast (LK2G12) employed in these studies is the same one used in previous investigations (2, 3, 4, 5) on galactose adaptation. It is a diploid strain, a representative of *S. cerevisiae*, possessing the physiological characteristics typical of the baking-type yeasts. For the purposes of these experiments, which require relatively large amounts of yeast, the culture was grown for 24 hours at 30°C. in 9-l. pyrex flasks, each containing 5 l. of medium, with constant and vigorous aeration through porous alundum stones. The medium employed was of the following composition: 100 g. hexose (glucose or galactose), 100 g. corn steep liquor (obtained through courtesy of Anheuser-Busch, St. Louis), 20 g.  $(\text{NH}_4)_2\text{SO}_4$ , 2.5 g.  $\text{NH}_4\text{H}_2\text{PO}_4$ , 10 ml. yeast autolyzate, and tap water to make 5 l. This was sterilized by autoclaving, the hexose being sterilized separately in a 20% solution and subsequently added to the other components.

The harvested yeast was dried overnight at room temperature, in a thin layer on glass plates in front of an electric fan.

Lebedew juices were prepared according to Neuberg and Lustig (11) by extracting the dried yeast (after grinding in the cold, either in a ball mill or in a chilled mortar) with 6 volumes of  $M/15$   $(\text{NH}_4)_2\text{HPO}_4$  for 3 hours at 37°C., after which the residue was removed by centrifugation at 5000 r.p.m. for about 5 minutes. In most experiments, 0.5 ml. of such a preparation were used per Warburg vessel in a total volume of 2 ml. of liquid. The pH of the extracts was adjusted to 6.2 whenever necessary. In agreement with Neuberg and Lustig, we found extracts with  $\text{Na}_2\text{HPO}_4$  generally inactive.

All measurements were made at 30°C. with Warburg manometers shaken at a rate of about 110 complete oscillations per minute. In all cases,  $\text{CO}_2$  production was determined in an atmosphere of  $\text{N}_2$ .

In testing for enzymatic activity, reagent grade glucose was used. Difco's purified galactose was further treated according to a method previously described (3).

When coenzyme, without further specification, is referred to in what follows, a boiled yeast juice is intended. This was prepared by adding 1 g. of dried yeast or 3 g. of fresh yeast to 10 ml. of water, boiling for a few minutes, and centrifuging down the residue of denatured protein. The clear supernatant is used as the source of coenzymes.

Coenzyme I was prepared by the method of Williamson and Green (12).

Hexose diphosphate was obtained as the commercial barium salt, Schwarz Laboratories, N. Y., which was converted to the potassium salt for use.

### EXPERIMENTAL

#### *a. The Enzymatic Activity of Lebedew Juices*

Before proceeding to the separation of the enzyme systems into apoenzyme and coenzyme fractions, a study of the intact enzyme systems in cell-free Lebedew preparations was undertaken. These experiments had two primary purposes. One was to compare the behavior of such extracts prepared from adapted and unadapted cells upon the

addition of glucose and galactose. The other purpose was to obtain some preliminary information on the completeness of these preparations with respect to cofactors. Insofar as known cofactors are concerned, the ones which received primary consideration were hexose diphosphate (HDP) and Coenzyme I.

Table I summarizes the pertinent information obtained. The rates recorded are those attained after 150 minutes, when the fastest combinations had reached a constant rate.

TABLE I

*Behavior of Lebedew Preparations*

The numbers represent mm.<sup>3</sup> of CO<sub>2</sub> evolved/0.5 cc. of extract/hour. The results recorded were all obtained on a single pair of preparations. They are typical of those obtained with other extracts during the course of the investigation. The final concentration of hexose diphosphate (HDP) was  $2.5 \times 10^{-3} M$ , of Coenzyme I,  $5 \times 10^{-4} M$ , and of glucose and galactose 3%. The amount of boiled yeast juice was 0.1 cc. in a total volume of 2 cc. of fluid; the galactose-grown extracts received unadapted juice, while the glucose-grown extracts received adapted juice.

Lebedew preparation from cells grown on	Substrate	No additions	Boiled yeast juice	Boiled yeast juice +HDP	HDP +Co. I	Boiled yeast juice +HDP +Co. I
Galactose	Glucose	3	7	750	240	1170
	Galactose	2	3	560	600	1270
	No substrate	—	—	80	25	98
Glucose	Glucose	3	5	280	220	620
	Galactose	1	4	80	30	101
	No substrate	—	—	75	38	112

The extremely low rates obtained in the absence of any additions indicate that all these preparations are incomplete. Supplementing them with coenzyme in the form of boiled yeast juice results in no significant change in activity, as seen by the values listed in Column 4. Our Lebedew extracts and boiled yeast juice are as poor in hexose diphosphate as are most preparations of this kind. This is evident from the effect of its addition as illustrated by the rate in Column 5. Note that the addition of this compound did not raise the galactose-fermenting capacity of unadapted extracts significantly above the endogenous rate, which appears immediately below it.

The use of hexose diphosphate to shorten the induction period is essential in such experiments to avoid the results of bacterial con-



tamination in prolonged experiments. Experiments lasting more than 3 hours must be controlled by bacteriological analysis to insure the significance of the manometric data obtained, for Lebedew extracts are excellent culture media.

Lebedew preparations are often low in Coenzyme I, which suggests the possibility that this is the component in the boiled yeast juice which is responsible for its activating effect. To test this, Coenzyme I was added in excess ( $5 \times 10^{-4} M$ ) in place of the boiled yeast juice in one set (Column 6), and another (Column 7) received both.

If the addition of boiled yeast juice is merely equivalent to adding Coenzyme I, adding it to a preparation already containing it in excess should not lead to a further increase in rate. If, however, the boiled yeast juice contained factors other than Coenzyme I, which were necessary for fermentation and were present in limiting concentrations, then the addition of the boiled yeast juice should result in stimulation. It is clear, comparing corresponding values in Columns 6 and 7, that the results point to the second possibility mentioned above. In all cases the addition of the boiled yeast juice to the extracts already containing adequate amounts of Coenzyme I led to significant increases in rate.

From a practical point of view these experiments permit the conclusion that the maximal activity of apozymase preparations derived from such extracts should be measured in the presence of HDP, of boiled yeast juice, and of excess Coenzyme I. This was done in all experiments subsequently described.

It is noteworthy that the rate of glucose fermentation by galactose-grown yeast is almost twice as great as the corresponding rate attained by extracts of glucose-grown yeast. This has been observed consistently, not only in Lebedew juice but, as will be seen below, in apozymases prepared in various ways. Since no such difference is found when intact galactose- and glucose-grown cells are compared, it is probable that adaptation by growth in galactose so changes the yeast that the glucozymase becomes either more stable or more easily extractable.

There are several conclusions of theoretical interest which follow from these results. From a comparison of Columns 5 and 7 it is clear that Coenzyme I stimulates adapted Lebedew extract whether it be fermenting glucose or galactose. Further, HDP can abolish the lag

period whether such an extract is fermenting glucose or galactose. It is, therefore, probable that the mechanisms of glucose and galactose fermentation by an adapted extract have at least those steps in common which involve these cofactors. It is likely from the work of previous investigators (13) that galactose is converted to glucose or one of its phosphorylated intermediates in the process of its fermentation. Kosterlitz (14) has suggested that the formation of a galactose-1-phosphate is the first step of the adaptive system. This is then presumably converted to glucose-1-phosphate by another enzymatic component present only in adapted cells.

More important for the basic issues of the present paper is the fact that, if the rates in Table I are corrected for the endogenous fermentation rates recorded in the row marked "no substrate," the ability of Lebedew extracts from unadapted yeast to ferment galactose is seen to be zero, no matter what additions are made. At the same time, such extracts have no difficulty in handling glucose. On the other hand, extracts derived from adapted cells ferment both glucose and galactose equally well. From this we can draw the theoretically important conclusion, in agreement with previous results (5, 6) using cell-free extracts obtained with different procedures, that an enzyme system which is unobtainable from unadapted cells can easily be prepared from adapted ones.

### *b. Preparation of Apoenzymes*

Attempts were made to prepare apoenzymes by the classical procedure of repeatedly washing dried yeast with water or buffer until all cofactors are washed out, as was done by Euler and Jansson (10). These attempts were unsatisfactory, as has commonly been found with baker's yeast. Inactivation of the apoenzyme invariably accompanied adequate extraction of cofactors. By stopping the washing procedure before complete cofactor extraction was attained, it was possible to obtain preparations which gave results similar to those of Euler and Jansson (10), but with inacceptably high blanks. Attempts to get around this difficulty by using the  $\text{CCl}_4$  extraction method of Govier (15) resulted in uniformly inactive preparations.

We accordingly attempted to convert the Lebedew extract from the dried yeast into a cofactor-free system. Two methods have been employed successfully. In the first to be described, the ability of activated charcoal to adsorb organic molecules was used to free the juice of cofactors. It is known from the work of Jandorf (16) that Coenzyme I among others is efficiently removed by this means.

The Lebedew juice was shaken with activated charcoal (Merck USP) for several minutes at room temperature. The charcoal was then removed by centrifugation, and residual traces of charcoal filtered out. It was found that 4 such treatments were adequate to produce an extract which was completely inactive in the absence of added cofactors. Preparations made from adapted and unadapted cells in this manner were then tested for the ability of adapted and unadapted coenzyme (boiled yeast juice) to activate them to the fermentation of both glucose and galactose.

Typical results are summarized in Table II. The rates obtained in the presence of added substrate (in rows labeled "Glucose" and "Galactose") must be corrected by subtracting the corresponding endogenous readings given in the row labeled "No substrate." The last row, in which glucose but no coenzyme was added, checks the adequacy of the preparative method. It is clear from the low values that coenzymatic factors critically necessary for the activity of these preparations have been removed.

TABLE II

*Apoenzymes Prepared by Charcoal Adsorption*

The numbers represent mm.<sup>3</sup> of CO<sub>2</sub> evolved/hour/0.5 cc. of extract. All cups received 0.1 cc. of Co. I and 0.1 cc. HDP supplementary to the additions noted. All concentrations as in Table I.

Substrate	Adapted apoenzyme		Unadapted apoenzyme	
	Unadapted coenzyme	Adapted coenzyme	Unadapted coenzyme	Adapted coenzyme
Galactose	1580	1500	80	96
	1490	1520	68	114
Glucose	1650	1710	480	511
	1470	1590	600	550
No substrate	180	230	72	90
Glucose without coenzyme	23		5	

Examination of the activities obtained on the addition of galactose reveals that only the adapted apoenzyme-containing system possesses measurable ability to ferment this hexose. When corrections for the endogenous fermentation are made, the activity of the adapted apoenzyme is about 1300, as compared with only 10 for the unadapted.

The data in the glucose row indicate that either one of the coenzymes is equally utilizable in the fermentation of glucose by both types of apoenzymes. This fact alone might imply either that the adapted coenzyme is completely unchanged, or that, if it has been modified, the modification consists only in the addition of a new component to the ones already present. However, the fact that either coenzyme was equally capable of activating the adapted apoenzyme clearly proves that the adaptation does not involve a modification in the coenzyme portion of the system. That some modification in the apoenzyme occurs during enzymatic adaptation is manifest from the fact that fermentability is always found associated with apoenzymes derived from adapted cells.

In evaluating the significance of these findings, it must be realized that the charcoal method of preparing an apoenzyme does not necessarily yield an absolute apoenzyme in the sense that the resulting preparation is completely free of all diffusible factors. Necessarily the only ones removed are those that are adsorbed on charcoal. These experiments therefore do not completely exclude the possibility that the change during adaptation is solely a coenzymatic one. The diffusible factor involved would, however, have to be non-adsorbable on charcoal, and also heat labile in view of the inability of adapted coenzyme to activate unadapted apoenzyme.

To test this possibility apoenzymes were prepared by a dialysis procedure. This has the advantage that the removal of the coenzymatic components depends only on their distinguishing characteristic, *i.e.*, diffusibility. Collodion membranes cast in 15 cc. test-tubes with Merck's ether-alcohol collodion solution were employed. Our enzyme preparations did not survive the protracted periods required by the usual dialysis methods. Accordingly, a procedure devised by M. F. Utter (private communication) which results in unusually rapid dialysis was employed. The effectiveness of this method is primarily due to the fact that both the inside and outside solutions are stirred continuously during the process.

The agitation of the inside fluid is accomplished by leaving an air space in filling the collodion tube, which is then rotated in an inclined plane, thus causing the air space to traverse the length of the tube periodically. This kind of rotation is accomplished by fastening the collodion tube along the cross-arm of a T-shaped glass rod mounted in a motor held at an angle of 45°. The rod is rotated at about 60 r.p.m. in a 10-gallon crock filled with *M*/15 phosphate buffer at pH 6.2. The entire dialysis was

carried out in a cold room held at 2°C. Using this procedure, adequate apoenzymes could be prepared with from 3–4 hours of dialysis. Prolongation of the dialysis beyond this period led to drastic loss of activity.

The results obtained with such apoenzymes are summarized in Table III. The adequacy of the removal of the cofactors is illustrated by the low values obtained in the absence of coenzyme, as shown in the last row. The endogenous rates observed when coenzyme is added are higher, and are probably due to the presence of fermentable substrate in the coenzyme preparations. When allowance is made for these rates, it is seen that the same situation holds on adding glucose and galactose as obtained in the apoenzymes prepared by charcoal treatment.

TABLE III

*Apoenzymes Prepared by Dialysis*

The numbers represent mm.<sup>3</sup> of CO<sub>2</sub> evolved/hour/0.5 cc. of extract. All cups received 0.1 cc. of both Co. I and HDP supplementary to the additions noted. All concentrations as in Table I.

Substrate	Adapted apoenzyme +unadapted coenzyme	Unadapted apoenzyme +adapted coenzyme
Galactose	351	38
	387	45
Glucose	375	178
	425	163
No substrate	24	39
Glucose without coenzyme	4	6

Adapted apoenzyme can use unadapted coenzyme equally well for both glucose and galactose fermentation. Further, coenzyme derived from adapted cells confers no measurable capacity to ferment galactose on the unadapted apoenzyme. The latter can, however, use the adapted coenzyme to ferment glucose. Here again we may note that the glucosylase activity of the unadapted apoenzyme is lower than that of the adapted one.

## DISCUSSION

These experiments provide some of the data necessary to decide on a plausible mechanism for the adaptation by yeast cells to the fermentation of galactose.

The suggestion that adaptation might represent the time required for the accumulation of special intermediates foreign to the glucosylase complex (*e.g.*, phosphorylated galactose) seems implausible on the face of it. It is difficult to see how such intermediates could accumulate without the previous synthesis of the requisite enzymes, necessary for their formation. One would have to assume, as Sevag (17) does, that an enzyme like hexokinase is non-specific and could phosphorylate galactose as well as glucose. Sevag offers in support of this supposed non-specificity the fact that hexokinase can phosphorylate mannose and fructose as well as glucose. He fails, however, to recognize that these three hexoses have a common enol form not shared by galactose, whose fundamental difference from them exists at the fourth carbon. In any case, the recent work of Kunitz and McDonald (18) specifically disposes of this suggestion, since these authors have found that crystalline hexokinase possesses no ability to catalyze the phosphorylation of galactose.

Hypotheses which seek to account for adaptation in terms of quantitative modifications of already existent components of the glucosylase system meet with grave difficulties of a biological nature which have apparently been disregarded by Sevag (1946). Many strains of yeast which possess a functional glycolytic system never acquire the ability to ferment galactose, no matter how long they be transferred in its presence. Obviously the simple possession of the ability to ferment glucose, and consequently of all the necessary enzymes, coenzymes, and intermediates, by no means guarantees the capacity to adapt to galactose fermentation. Furthermore, it is well known from previous studies by Lindegren (19) that this capacity to adapt to galactose is inherited in a Mendelian fashion and is apparently controlled by a single gene. One would, therefore, have to argue that the genetic difference between a galactose-adaptable strain and a non-adaptable strain is reflected only in a quantitative variation of one or more of the glucosylase components common to each. A conclusion of this kind would be in serious disagreement with recent work, which clearly indicates that, wherever the examination has been possible, genetic differences invariably lead to demonstrable, qualitative differences in enzymatic constitution.

Insofar as the present experiments are concerned, the attempt to explain adaptation by ascribing it to action of some vaguely-defined

non-specific enzymatic component of the glucozymase system is flatly excluded by the results with dialyzed apoenzymes. The same conclusion applies to theories involving the level of glucozymase cofactors or intermediates. This method of apoenzyme preparation would remove not only the coenzymes but also the intermediates. If the sole difference between Lebedew extracts from adapted and unadapted cells resided in the fact that the former contain intermediates not present in the latter, or contain higher concentrations of compounds present in both, dialysis should convert an adapted Lebedew extract into an unadapted one. It is clear from the results in Table III that this is not the case. Dialyzed Lebedew extract derived from galactose-adapted cells retains the capacity to ferment galactose when combined with Coenzyme I, HDP, and boiled yeast juice from unadapted cells. Since none of these additions could conceivably contain intermediates peculiar to galactose-fermenting cells, it is obvious that these alone can play no role. In view of this, it seems necessary to look to enzyme changes for an explanation of the adaptive process.

The experiments with both types of apoenzymes appear to offer unequivocal answers as to what type of enzymatic change has occurred, *i.e.*, whether apoenzymes, coenzymes, or both were involved. We may summarize the results obtained with all combinations of adapted and unadapted apoenzymes and the corresponding coenzymes by the following:

- (1) Adapted apoenzyme + unadapted coenzyme = adapted enzyme;
- (2) Adapted apoenzyme + adapted coenzyme = adapted enzyme;
- (3) Unadapted apoenzyme + unadapted coenzyme = unadapted enzyme;
- (4) Unadapted apoenzyme + adapted coenzyme = unadapted enzyme.

It is evident from these results that the capacity to ferment galactose is invariably associated with the apoenzyme derived from adapted cells. It is, therefore, difficult to avoid the conclusion that it is the apoenzyme or protein component of the enzyme complex which is modified during the course of adaptation. That only this modification is involved follows from the ability of unadapted coenzyme equivalently to replace adapted coenzyme in activating the adapted apoenzyme to galactose fermentation.

## SUMMARY

The ability of Lebedew extracts from galactose-adapted yeasts to ferment galactose was demonstrated. Similar preparations from unadapted cells failed to do so.

The question of whether adaptation involves the formation of apoenzymes or of coenzymes was investigated by preparing adapted and unadapted forms of both. Combining these in all possible ways, it was found that the activity was invariably associated with the adapted apoenzyme. In view of these results, it is concluded that the change by which a cell becomes capable of fermenting galactose is a modification in the apoenzymatic component of the enzyme system.

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**The Synthesis of S-Carboxymethylhomocysteine  
and S- $\beta$ -Carboxyethylhomocysteine and a  
Study of Their Availability for Growth  
of Rats Maintained on a Low-  
Casein Diet**

**Jakob A. Stekol\***

*From the Department of Chemistry, Fordham University, New York 58, N. Y.,  
and the Department of Biochemistry, Vanderbilt University  
Medical School, Nashville, Tenn.*

Received January 22, 1947

**INTRODUCTION**

Neither S-methylcysteine (1), S-carboxymethylcysteine (2), nor S-ethylhomocysteine (ethionine) (3) is available to rats for growth purposes in lieu of cystine or methionine. It appeared of interest to investigate the availability of S-carboxymethylhomocysteine and carboxyethionine (S- $\beta$ -carboxyethylhomocysteine) to rats for growth purposes. As far as we are aware, these two derivatives of homocysteine have not been previously prepared.

The compounds were prepared by demethylation of methionine in liquid ammonia with metallic sodium followed by the addition of either  $\beta$ -bromopropionic acid or monochloroacetic acid to the reaction vessel. Although homocystine could also be used instead of methionine as the starting material, the direct demethylation of methionine offers definite advantages from the standpoint of time, labor and yields. The same method was used by us previously in the preparation of S-benzylhomocysteine (4).

\* The data presented here were obtained in 1940-1942. The publication was delayed by service overseas during the war. Present address: Amino Products Research Division, International Minerals and Chemical Corporation, Rossford, Ohio.

## EXPERIMENTAL

*S*-Carboxymethylhomocysteine. Ten g. of *dl*-methionine were dissolved in about 150 ml. of liquid ammonia and about 3 g. of clean metallic sodium were added in small portions to the solution with constant stirring of the reaction mixture. Six and four-tenths g. of powdered monochloroacetic acid were added to the reaction mixture in small portions. The reaction is quite violent. After all the acid had been added, ammonia was allowed to evaporate and the reaction vessel was evacuated to remove as much ammonia as possible. To the residue, 50 ml. of cold water were added, the solution was filtered if necessary, the filtrate was chilled on ice and the pH of the solution was adjusted to 3.5–4.0 with HCl. The crystallized material was removed by filtration after standing on ice for several hours, washed with cold water, then recrystallized 3 times from hot water. After drying at 110°C., the product melted at 218–220°C. (uncorrected). Upon recrystallization from water, the m.pt. remained unchanged. The yields of pure product were 65–70% of the theoretical amount. Under the microscope the material had the appearance of sharp needles.

The product gives a positive ninhydrin and a negative nitroprusside test either before or after treatment with sodium cyanide. It is somewhat soluble in cold water, much more so in hot water or dilute alkali. It is insoluble in ethanol, methanol, or ethyl ether. The analytical values obtained on the material which was dried at 100°C. *in vacuo* over  $P_2O_5$  agreed reasonably well with those calculated for *S*-carboxymethylhomocysteine.

	C	H	N	S
Found	37.17	5.56	7.60	16.32
Calculated for $C_6H_{11}NO_4S$	37.31	5.70	7.25	16.59

*S*- $\beta$ -carboxyethylhomocysteine. A similar procedure to that described above was used in the preparation of this compound from 10 g. of *dl*-methionine and 10.3 g. of  $\beta$ -bromopropionic acid. The dried product melted with decomposition at 221–222°C. (uncorrected), and, on recrystallization from water, the m.pt. remained unchanged. The nitroprusside test on the material was negative either before or after treatment with sodium cyanide, and its solubility properties were

qualitatively similar to those of S-carboxymethylhomocysteine. The analytical results are shown below.

	C	H	N	S
Found	40.80	6.56	6.59	15.03
Calculated for $C_7H_{13}NO_4S$	40.58	6.28	6.76	15.46

### FEEDING EXPERIMENTS

Male albino rats, born and raised in the laboratory, were used. The basal diet consisted of Labco casein 5, corn starch 51, sucrose 15, inorganic salts 4 (5), powdered brewer's yeast 5, corn oil 15, and cod liver oil 5%. The supplements to this diet were added in the following amounts: choline chloride 0.5, *L*-cystine 0.5, *DL*-methionine 0.63, S-carboxymethylhomocysteine 0.83 or 1.66, and S- $\beta$ -carboxyethylhomocysteine 0.87%. Food and water were allowed *ad libitum*. The rats were weighed twice a week and a record of food consumption was made.

### RESULTS

The data obtained are summarized in Table I. Neither S-carboxymethylhomocysteine nor S- $\beta$ -carboxyethylhomocysteine stimulated the growth of rats under the condition under which either *L*-cystine or *DL*-methionine is effective. It should be noted, however, that in contrast to S-ethylhomocysteine (3), S- $\beta$ -carboxyethylhomocysteine does not, apparently, produce toxic symptoms which lead to death of the growing rat. The metabolic path of the two substituted homocysteines presented here is not known at present. It may be conjectured, however, on the basis of the results obtained, that neither substance is converted *in vivo* to homocysteine or cystine in amounts sufficient for the stimulation of growth under the conditions employed, if at all.

### SUMMARY

1. S-Carboxymethylhomocysteine (carboxymethionine) and S- $\beta$ -carboxyethylhomocysteine (carboxyethionine) were synthesized and characterized.

2. Neither substance is available for growth of rats maintained on a low-casein diet.

TABLE I

*The Effect of S-Carboxymethylhomocysteine and S-β-carboxyethylhomocysteine on the Growth of Rats Maintained on a Low-Casein Diet*

Rat No.	Initial weight	Total gain	Days on diet	Food intake per day	Supplement/100 g. diet
	g.	g.		g.	mg.
23 ♂	38	15	21	6.2	500 Choline Cl
	53	14	21	5.6	Same plus 830 CMH
	67	17	21	7.7	500 Choline Cl
27 ♂	36	17	21	6.0	500 Choline Cl
	53	18	21	8.0	Same plus 870 CEH
	71	20	21	8.9	500 Choline Cl
29 ♂	54	16	21	5.5	500 Choline Cl
	70	18	21	8.8	Same plus 1660 CMH
	86	17	21	8.9	500 Choline Cl
18 ♂	45	11	21	4.4	500 Choline Cl
	56	35	21	6.8	Same plus 630 M
	91	8	21	8.0	500 Choline Cl
16 ♂	50	13	21	4.9	500 Choline Cl
	63	38	21	8.8	Same plus 600 C
	101	10	21	9.3	500 Choline Cl
32 ♂	44	33	65	6.0	500 Choline Cl

The data on each rat are representative of experiments obtained on 4 animals. In the last column the letters CMH denote S-carboxymethylhomocysteine; CEH, S-β-carboxyethylhomocysteine; M, *dl*-methionine; and C, *l*-cystine.

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# The Enzymatic Conversion of Tryptophan to Auxin by Spinach Leaves

S. G. Wildman, Mario G. Ferri\* and James Bonner

*From the William G. Kerckhoff Laboratories of the Biological Sciences,  
California Institute of Technology, Pasadena*

Received January 31, 1947

## INTRODUCTION

A substantial body of evidence now indicates that indoleacetic acid may be a widespread natural auxin. Thus free indoleacetic acid has been isolated in pure form from immature corn seeds by Haagen-Smit *et al.* (6, 7) and qualitative tests indicating the similarity of natural auxins to indoleacetic acid have been reported by many workers (4, 7). In this paper it will be shown that a typical plant tissue, the spinach leaf, possesses an enzyme system for the production of auxin, presumably indoleacetic acid, from tryptophan.

The first indication that tryptophan might act as a precursor of auxin was Thimann's demonstration that *Rhizopus Suinus* forms IAA\*\* in the presence of tryptophan under conditions favorable to oxidative deamination (15). Skoog (13) has shown that tryptophan is active in the decedded *Avena* coleoptile. Indolethylamine also appears to be converted into an active auxin by the coleoptile. Skoog, who believed the native auxin of *Avena* to be auxin *a* did not, however, consider tryptophan to be a natural precursor of auxin in *Avena*.

Berger and Avery (3, 4) have isolated from corn seeds a substance, itself inactive, from which auxin can be obtained by autoclaving under alkaline conditions. Enzyme extracts prepared from corn or *Avena* coleoptiles are not able to activate the material. Although this substance was not obtained in pure form, Berger and Avery did indicate that the active product formed is IAA. Since tryptophan is converted to IAA to an inappreciable extent by heating with alkali, Avery and Berger (1) have concluded that tryptophan is not the compound from which auxin is liberated by treatment of corn seeds.

The evidence to be presented below indicates that tryptophan may serve as a precursor of auxin in living plant tissue.

\* Permanent address: Faculdade de Filosofia, Ciencias e Letras, Universidade de São Paulo, Brasil.

\*\* IAA will represent indoleacetic acid throughout the balance of this paper.

## METHODS AND MATERIALS

*Auxin Determinations.* The standard *Avena* method (Went and Thimann, 18) was used for auxin measurements. Only curvatures within the proportionality range of dilution were used for calculation of results. Sensitivity of the test plants was determined for each experiment with crystalline IAA and all results are expressed as curvatures and computed in terms of IAA. Unknown solutions to be tested for auxin activity were acidified to pH 3 (glass electrode), extracted with freshly distilled, peroxide-free ether and treated in the manner described by Wildman and Gordon (19).

Twice recrystallized, *l*-tryptophan was used in this study. Such material is known to be inactive in the standard *Avena* test, although a delayed response may develop after the 90 minutes reaction time customarily used in the *Avena* assay (18).

All enzyme experiments were protected against bacterial contamination by a layer of toluene and additionally by relatively short incubation periods.

*Protein Preparations.* Cell-free extracts were prepared from fresh spinach leaves by the methods of Wildman and Bonner (20). *Whole protoplasm* as used here signifies lyophilized leaf tissue from which only the cell walls have been removed. *Whole cytoplasm* signifies whole protoplasm minus the chloroplasts. *Whole dialyzed cytoplasm* is whole cytoplasm which has undergone dialysis against several changes of 0.01 *M* phosphate buffer, pH 6.8.

## EXPERIMENTAL RESULTS

*Conversion of Tryptophan to Auxin by Living Spinach Leaves*

The following experiment shows that spinach leaves are capable of forming from tryptophan a substance highly active in the *Avena* test.

Leaf discs were cut from fresh, turgid spinach leaves with a sharp cork borer. Each disc was then divided into halves with a razor blade. One-half of the discs was placed in 10 ml. of 0.1 *M* phosphate buffer, pH 7.0, while the other half was placed in the same volume of buffer containing 10 mg. of tryptophan. Both lots were then infiltrated with solution by repeated evacuation, after which the discs were allowed to incubate in air for 3.5 hours at room temperature (24°C.). At the end of this time, the leaves were thoroughly washed with distilled water, rapidly dried at 80°C., and coarsely ground. The dry, ground leaf samples were then extracted one hour with ether and the ether extracts tested for auxin activity.

Data in Table I indicate that the leaf segments which had been infiltrated with tryptophan contained 10 times more auxin than the buffer-infiltrated controls. This experiment was repeated with longer incubation periods and with smaller concentrations of tryptophan with similar results.

From these observations, it can be concluded that spinach leaves contain a mechanism for the production of an auxin in the presence of tryptophan.

TABLE I

*The Conversion of Tryptophan to Auxin by Living Spinach Leaves*

10 ml. of 0.1 M  $\text{KH}_2\text{-K}_2\text{HPO}_4$  buffer, pH 7.0; temperature 22°C. Leaves infiltrated by placing in vacuum for 20 minutes. Incubation in air.

Infiltration medium	Time	Dry weight of leaves in	Curvature in degrees	Agar	IAA $\times 10^{-5}$ mg./100 mg dry leaves
	hrs.	mg.		ml.	
Buffer	3.5	95	$6.4 \pm 0.5$	0.80	2.5
Buffer+10 mg. tryptophan	3.5	89	$13.6 \pm 1.4$	3.20	27.5
Buffer	5	64	$3.5 \pm 0.4$	0.80	3.7
Buffer+2.5 mg. tryptophan	5	63	$9.0 \pm 1.1$	6.40	39.4

*Conversion of Tryptophan to Auxin by Cell-Free Extracts*

The following experiment shows that the conversion of tryptophan to auxin by spinach leaves is an enzymatic process. Whole protoplasm and whole dialyzed cytoplasm were incubated with or without added tryptophan. In each case, a control was run in which enzyme activity was destroyed by heat denaturation of the proteins before addition of the tryptophan. From the results, presented in Table II, it is evident that tryptophan is rapidly converted to an auxin by cell-free spinach

TABLE II

*Conversion of Tryptophan to Auxin by Lyophilized, Cell-Free Extracts of Spinach Leaves*

7.0 ml. of 0.1 M  $\text{KH}_2\text{-K}_2\text{HPO}_4$  buffer, pH 6.8. Time, 3.5 hours. Temperature 22°C. Enzyme destroyed by heating at 90°C. for 20 minutes.

Enzyme preparation	Condition	Tryptophan added	Curvature in degrees	Agar	IAA $\times 10^{-5}$
		mg.		ml.	mg.
50 mg. whole protoplasm	Not heated	0	0	0.80	0
50 mg. whole protoplasm	Heated	5.0	0	0.80	0
50 mg. whole protoplasm	Not heated	5.0	$8.4 \pm 2.0$	1.60	25
25 mg. whole dialyzed cytoplasm	Not heated	0	0	0.80	0
25 mg. whole dialyzed cytoplasm	Heated	5.0	0	0.80	0
25 mg. whole dialyzed cytoplasm	Not heated	5.0	$4.2 \pm 0.7$	1.60	13



leaf extracts. Auxin activity was formed in the presence of tryptophan by the unheated preparation. It was not formed by preparations which had been heated to destroy enzyme activity, nor was it formed in reaction mixtures from which tryptophan was omitted. Concordant results were obtained with many other preparations from different spinach leaves.

*The Effect of Oxygen on the Conversion of Tryptophan to Auxin*

The mechanism involved in the conversion of tryptophan to auxin may be oxidative in nature as indicated by the fact that auxin formation is greatly reduced when the reaction mixture is incubated in the absence of oxygen. Thunberg tubes were employed for the experiment so that tryptophan could be added to the enzyme preparation after oxygen was removed by evacuation of the vessels with a water aspirator. Whole cytoplasm was used as enzyme. Table III shows that auxin formation was reduced by approximately 80% in the evacuated tubes. Similar results were obtained with a second spinach leaf preparation.

TABLE III

*Effect of Oxygen on the Conversion of Tryptophan to Auxin*

Conditions the same as in Table II. Vacuum produced by a water aspirator.

Enzyme preparation	Condition	Curvature in degrees	Agar	IAA $\times 10^{-5}$
			ml.	mg.
100 mg. whole cytoplasm	Air	$8.4 \pm 0.9$	3.20	13
100 mg. whole cytoplasm	Vacuum	$5.5 \pm 0.5$	1.60	4
100 mg. whole cytoplasm	Air-Boiled	$0.9 \pm 0.4$	1.60	0.6

*The Effect of Keto-Fixatives on the Conversion of Tryptophan to Auxin*

Since oxygen appears to be required for the enzymatic conversion of tryptophan to auxin, it is possible that the oxidative mechanism suggested by Thimann (15) for *Rhizopus* may also apply to the spinach leaf. Thimann proposed that tryptophan might first be converted by oxidative deamination to indolepyruvic acid (IPA) which would then be decarboxylated and oxidized to IAA.

To test the hypothesis that conversion of tryptophan involves a keto compound as an intermediate, use was made of two substances

known to add on to carbonyl groups as keto-fixatives. Tryptophan was incubated with whole dialyzed cytoplasm and buffer containing various amounts of either sodium bisulfite or sodium cyanide. The results of one experiment are recorded in Table IV. Tryptophan conversion to

TABLE IV

*Effect of Keto Fixatives on the Conversion of Tryptophan to Auxin*

23 mg. of whole dialyzed cytoplasm incubated 3.5 hours with 2.5 mg. of tryptophan. Other conditions the same as given in Table II.

Poison	Final molarity of reaction mixture	Curvature in degrees	Agar	IAA $\times 10^{-4}$
			ml.	mg.
None	—	10.0 $\pm$ 0.6	0.80	3
NaCN	10 <sup>-2</sup>	0	0.80	0
NaCN	10 <sup>-3</sup>	0	0.80	0
NaCN	10 <sup>-4</sup>	2.1 $\pm$ 0.7	0.80	0.5
NaHSO <sub>3</sub>	10 <sup>-2</sup>	0	0.80	0
NaHSO <sub>3</sub>	10 <sup>-3</sup>	0	0.80	0
NaHSO <sub>3</sub>	10 <sup>-4</sup>	6.5 $\pm$ 0.6	0.80	2

auxin was completely inhibited by either 10<sup>-2</sup> or 10<sup>-3</sup> *M* cyanide or bisulfite. With 10<sup>-4</sup> *M* cyanide, a small amount of conversion took place and a still greater conversion occurred in the presence of bisulfite of the same strength. From these results, it appears probable that auxin formation from tryptophan may involve the formation of a keto compound. However, several attempts to regenerate an active substance from a bisulfite supplemented reaction mixture failed. It is also possible that cyanide may inhibit the reaction by not only combining with the keto intermediate, but also by directly inhibiting the enzyme.

#### *Indolepyruvic Acid as a Possible Intermediate in Tryptophan Conversion*

A logical keto intermediate in the reaction under consideration would be indolepyruvic acid. This compound was therefore infiltrated into spinach leaves.

Sixty leaf discs were removed from healthy leaves with a cork borer, washed, quartered with a razor blade and equally distributed into four beakers containing  $3.6 \times 10^{-3}$  mg. of IPA in 20 ml. of 0.1 *M* phosphate buffer, pH 4.5. The relatively low pH was chosen for this experiment because acids fail to penetrate into spinach cells at more alkaline pH values (5). The samples were placed under vacuum for 20 min-

utes, the vacuum released and the incubation continued in air at room temperature. One of the samples was immediately drained of liquid after releasing the vacuum, washed 5 times with 30 ml. portions of distilled water and rapidly dried at 80°C. Other samples were treated identically after 2, 4, and 8 hours incubation. All of the samples were coarsely ground, extracted with ether for one hour and the auxin activity of the ether extract determined. (Other experiments have consistently shown that an equal weight of spinach leaves infiltrated with buffer and incubated 4 hours under the same conditions contain auxin equivalent to  $2-3 \times 10^{-5}$  mg. of IAA.)

Data presented in Table V show that there was a progressive increase in the amount of extractable auxin up to 4 hours incubation with IPA. At 0 hours, auxin equivalent to  $8 \times 10^{-5}$  mg. IAA was extracted. After two hours,  $14 \times 10^{-5}$  mg. of IAA were found, the increase corresponding to 1.7% conversion of IPA into IAA. After 4 hours, an increase in auxin equivalent to a 9.4% conversion of IPA to IAA was found. The amount of auxin found after 8 hours dropped sharply but

TABLE V

*Effect of Infiltrating Living Spinach Leaves with Indolepyruvic Acid*

Leaf discs quartered and infiltrated by 20 min. vacuum treatment. Infiltration medium contained  $3.6 \times 10^{-3}$  mg. IPA in 20 ml. of 0.1 M  $\text{KH}_2\text{K}_2\text{HPO}_4$  buffer, pH 4.5. Temperature 21°C. Same weight of leaves infiltrated with plain buffer and incubated 4 hours contain  $2-3 \times 10^{-5}$  mg. IAA/100 mg. dry weight.

Time	Dry weight of leaves	Curvature in degrees	Agar	IAA $\times 10^{-5}$ /100 mg. dry weight of leaves	Per cent conversion
<i>hrs.</i>	<i>mg.</i>		<i>ml.</i>	<i>mg.</i>	
0	98	$5.5 \pm 0.8$	3.20	8	—
2	104	$10.7 \pm 0.6$	3.20	14	1.7
4	101	$9.1 \pm 1.2$	12.80	42	9.4
8	90	$8.1 \pm 1.0$	3.20	13	1.4

was still larger than the amount extracted at 0 hours. It is possible that prolonged periods of incubation at an acid pH may lead to the destruction of IAA, or possibly an inhibitor may be produced by prolonged incubation (see below). This experiment, which was repeated with similar results, indicates that, in the presence of living leaves, an active auxin may be produced from IPA. However, the exact nature of this auxin production is somewhat doubtful since, as will next be shown, IPA is itself unstable in solution.

*Behavior of Indolepyruvic Acid in Solution*

Kögl and Kostermans (8) first determined the activity of IPA in the *Avena* test and reported that IPA has about 1% of the activity of IAA. In the present experiments, IPA dissolved in phosphate buffer at pH 6.8 and then extracted with ether after acidification, had approximately 6% of the activity of IAA. However, treatment of the preparation in ether solution with activated charcoal resulted in a compound which possessed essentially no activity (no curvature from  $10^{-2}$  mg./0.8 ml. agar) in the *Avena* test when dissolved in buffer at pH 6.8, but which agreed in analysis, melting point ( $210^{\circ}\text{C}.$ ) and other properties with those expected for pure IPA.

*Analysis:*  $\text{C}_{11}\text{H}_9\text{O}_3\text{N}$ . Molecular weight, 203.1

Calculated: C, 64.99; H, 4.48; N, 6.89

Found: C, 65.4; H, 5.03; N, 6.04

However, it was noticed that the IPA solutions rapidly became orange-colored on standing for short periods of time suggesting that spontaneous oxidation of IPA might have occurred in solution.

To check on the stability of IPA in the acid solution used for the infiltration experiment,  $10^{-2}$  mg. of IPA were dissolved in 10 ml. of phosphate buffer at pH 4.5, the flask containing the solution stoppered and placed in a water bath at  $30^{\circ}\text{C}.$  One ml. aliquots were removed as soon as solution of IPA was complete (0 time), and after 1, 2, and 4 hours. It should be noted that the solution became strongly colored even before the acid was completely dissolved. The aliquots were quickly added to 10 ml. of phosphate buffer containing 20 mg. of NaCN at pH 9.0. After standing one-half hour to insure complete trapping of the keto acid, the solutions were acidified, extracted with ether and tested for non-cyanido-sensitive auxin activity by the *Avena* test. In the same experiment, it was observed that treatment with cyanide had no effect on the activity of IAA since IAA was quantitatively recovered from a cyanide mixture.

The results recorded in Table VI show that when IPA is dissolved in buffer at pH 4.5, it apparently spontaneously decomposes to products including IAA, since, at the time of complete solution (0 time), auxin activity equivalent to  $67 \times 10^{-5}$  mg. of IAA was found in the reaction mixture. This activity decreased with time as shown in Table VI. Whether this decrease in activity is the result of actual destruction of auxin or to the production of an inhibitor of the *Avena* test is at present unknown. No production of auxin activity occurred when  $10^{-2}$  mg. of IPA were dissolved in buffer at pH 6.8. Comparison of the results

TABLE VI

*Effect of Acid Buffer on the Stability of Indolepyruvic Acid*

IPA dissolved in phosphate buffer at pH 4.5 and incubated at 30°C. Aliquots removed after complete solution (0 time), and after 1, 2, and 4 hours incubation. The keto acid remaining was fixed with cyanide. See text for further details.

Time	Amount of IPA	Agar	Curvature in degrees	IAA $\times 10^{-5}$	Conversion of IPA to IAA
<i>hrs.</i>	<i>mg.</i>	<i>ml.</i>		<i>mg.</i>	<i>per cent</i>
0	10 <sup>-2</sup>	25.60	7.4 $\pm$ 0.7	67	6.7
1	10 <sup>-2</sup>	25.60	6.5 $\pm$ 0.5	58	5.8
2	10 <sup>-2</sup>	25.60	3.8 $\pm$ 0.6	34	3.4
4	10 <sup>-2</sup>	25.60	2.3 $\pm$ 0.5	21	2.1

presented in Table V with those recorded in Table VI suggests that the living leaf may influence the course and extent of auxin production from IPA, because it is apparent that a greater conversion occurred after 4 hours in the presence of leaves than can be accounted for by the spontaneous conversion of IPA alone.

It is interesting to note that the spontaneous decomposition of IPA involves both the absorption of oxygen and the liberation of carbon dioxide at pH 4.5, facts consistent with the idea of an oxidative decarboxylation of IPA to IAA.

One mg. of IPA was dissolved in 0.1 ml. of EtOH and placed in the side arm of a Warburg respirometer. Two ml. of phosphate buffer, pH 4.5, were placed in the main compartment and either KOH or water added to the center well depending upon whether O<sub>2</sub> uptake or CO<sub>2</sub> liberation was to be measured. After temperature equilibrium was reached the solutions were mixed. Until mixing, no pressure or color changes were noted, implying that IPA is stable in alcohol. After mixing, however, O<sub>2</sub> uptake and CO<sub>2</sub> evolution were rapid and the buffer became orange colored. In two hours, 49.5  $\mu$ l. of O<sub>2</sub> were consumed and 21  $\mu$ l. of CO<sub>2</sub> were evolved. From the ratio of O<sub>2</sub> consumed to CO<sub>2</sub> evolved, it is evident that the reaction is not simply the direct production of IAA from IPA according to the reaction,  $R-CH_2-CO-COOH + \frac{1}{2} O_2 \rightarrow R-CH_2-COOH + CO_2$ , where R represents the indole nucleus. Even if this reaction occurs, it must be accompanied by other oxidative processes. In a similar experiment performed at pH 6.8, 42  $\mu$ l. of oxygen were consumed by 1.0 mg. of IPA in 2 hours at 30°C. CO<sub>2</sub> evolution was not determined.

#### *The Effect of Cell-Free Extracts on IPA*

Although living spinach leaves appear to increase the production of auxin from IPA, it has not been possible to carry out a similar reaction

with enzyme preparations known to convert tryptophan to auxin. The results of a typical experiment are presented in Table VII. Various concentrations of the original sample of IPA prior to additional purification were incubated with whole dialyzed cytoplasm. Not only was the activity of the IPA not increased by incubation with undenatured enzyme preparations, but on the contrary, the initial activity of the reaction mixture appeared to decrease slightly during incubation, results paralleling those of Table VI. The experiment was repeated with the purified IPA with similarly negative results. This failure to obtain

TABLE VII

*Effect of Spinach Enzyme Extracts on Indolepyruvic Acid*

Incubation conditions the same as given in Table II.

Enzyme preparation	Condition	Indolepyruvic acid added	Curvature in degrees	Agar	IAA $\times 10^{-6}$
		mg.		ml.	mg.
100 mg. whole dialyzed cytoplasm	Heated	0	0	0.80	0
100 mg. whole dialyzed cytoplasm	Not heated	0	0	0.80	0
None	Not heated	$10^{-3}$	$11.0 \pm 1.0$	1.60	5
100 mg. whole dialyzed cytoplasm	Heated	$10^{-3}$	$5.2 \pm 0.5$	3.20	4
100 mg. whole dialyzed cytoplasm	Not heated	$10^{-3}$	$3.3 \pm 0.5$	3.20	3
100 mg. whole dialyzed cytoplasm	Heated	$10^{-4}$	$3.2 \pm 0.6$	1.60	1
100 mg. whole dialyzed cytoplasm	Not heated	$10^{-4}$	$1.1 \pm 0.5$	1.60	0.5
100 mg. whole dialyzed cytoplasm	Heated	$10^{-2}$	$4.7 \pm 0.4$	12.80	15
100 mg. whole dialyzed cytoplasm	Not heated	$10^{-2}$	$5.7 \pm 0.4$	6.40	9

*in vitro* conversion of IPA to a more active auxin may be attributable to the fact that IPA is also highly labile at the pH, 6.8, used in these experiments and is rapidly converted to an unknown colored derivative, possibly a polymer. More acid solutions such as used for the leaf infiltration experiment of Table V cannot be used with enzyme preparations, since the enzyme system for conversion of tryptophan to auxin is inactive at a more acid pH than 6.0 (Fig. 1).

#### *Tryptamine and Indoleacetaldehyde as Possible Intermediates*

An alternative mechanism for the conversion of tryptophan to indoleacetic acid would be *via* decarboxylation to tryptamine, deamination of this to indoleacetaldehyde followed by an oxidation to IAA. This idea finds support in the work of Larsen (9) who showed that

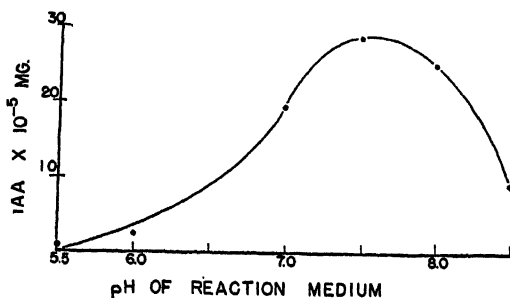


FIG. 1. Effect of pH on the Conversion of Tryptophan to Auxin. 100 mg. whole cytoplasm incubated with 2.5 mg. tryptophan contained in 7 ml. of 0.5 *M* phosphate buffer for 3.5 hours at 22°C.

indoleacetaldehyde is contained in etiolated peas and other plants and that this substance can be oxidized with enzyme preparations made from milk or soil organisms to a highly active auxin. However, spinach leaf sections are not able to form auxin at the expense of infiltrated tryptamine. Neither tryptamine nor indoleacetaldehyde incubated at various pH's with enzyme preparations known to be active in the conversion of tryptophan to auxin, were active in producing auxin activity. It would appear, therefore, that these compounds are not intermediates in the conversion of tryptophan to auxin by spinach leaves.

#### *Properties of the Tryptophan-Converting Enzyme*

Fig. 1 shows that the optimal pH for tryptophan conversion to auxin occurs in the region of pH 7-8. For these experiments, however, a pH of 6.8 was usually selected as more nearly representing the normal pH of expressed spinach juice. In Fig. 2 is recorded the progress of auxin appearance with time in a reaction mixture containing tryptophan and dialyzed whole cytoplasm at pH 6.8. After 4 hours of incubation at 24°C. a plateau is reached, even though the total fraction of the tryptophan converted to IAA is minute. This small total conversion may in part be owing to diversion of the intermediate IPA through other reactions as suggested above.

The tryptophan-converting enzyme may be concentrated by fractional ammonium sulfate precipitation of the whole cytoplasmic protein and the preparations so obtained may be lyophilized without immediate loss in activity. However, lyophilized preparations appear to lose activity on standing.

## COMMENTS

The experiments described above have demonstrated the presence of an enzyme system in spinach leaves which rapidly converts tryptophan into auxin. The evidence strongly suggests that tryptophan may be oxidatively deaminated to IPA which is then oxidatively decarboxylated to IAA. Because of the small yields and extremely small

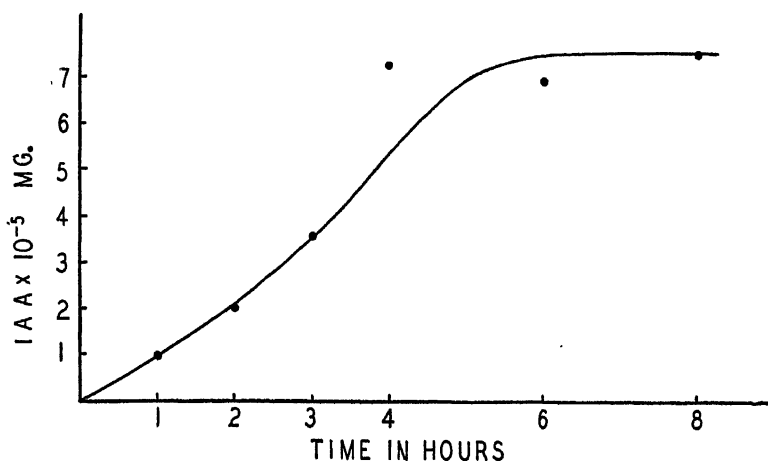


FIG. 2. Effect of Time on the Conversion of Tryptophan to Auxin. 23 mg. of whole dialyzed cytoplasm incubated with 2.5 mg. of tryptophan contained in 7 ml. of 0.01 M  $\text{KH}_2\text{K}_2\text{HPO}_4$  buffer, pH 6.8. Temperature 20°C.

quantities of reaction products formed, no attempt has yet been made to isolate either IPA or IAA as products of tryptophan conversion, but it is improbable that the final product can be other than IAA. Many indole compounds have been tested for their ability to cause curvatures of the *Avena* coleoptile, but none compare in activity with IAA and IAA is the only *Avena*-active indole compound to be expected from the degradation of tryptophan. Since the tryptophan-auxin converting mechanism has been found in all spinach leaf preparations so far examined, and since its substrate, tryptophan, is a universal plant constituent, it seems probable that IAA constitutes a native auxin in this plant. This view is supported by the low molecular weight and acid lability and alkaline stability of spinach leaf auxin (19).



As a precursor of auxin, tryptophan is readily available from the plant proteins as shown by the isotope experiments of Schoenheimer *et al.* (12) and Vickery *et al.* (17). These workers have found that amino acids are in dynamic equilibrium with proteins, *i.e.*, amino acids are continuously built into and released from proteins without the necessity of a net protein synthesis. Similarly, the synthesis of tryptophan from indole and serine found in *Neurospora* by Tatum and Bonner (14) may also apply to spinach leaves. Hence, it is conceivable that there is always a ready supply of tryptophan available in spinach leaves for conversion to auxin.

Numerous authors have described methods by which auxin can be formed or released from material which is inactive in the *Avena* test before treatment (11). Thus Avery, Berger and Shalucha (2) have shown that most of the auxin in corn seeds exists in a bound state from which IAA can be released by autoclaving under alkaline conditions. Wildman and Gordon (19) found that proteolytic enzymes release auxin from spinach leaf proteins. Thimann *et al.* (16) found that auxin extraction of plant tissues could be brought to a rapid end point when dry ether was used as a solvent, but like other workers (10), found that auxin was released at a small but continuous rate from plant material extracted with wet ether. It is possible that this auxin is also bound to protein from whence it is slowly released either by autolysis or by foreign proteolytic enzymes. While it might be presumed that such bound forms of auxin represent precursors, or perhaps storehouses of auxin, evidence presented by Wildman and Bonner (20) indicates that the bound auxin of spinach leaves is associated with a protein which is also an active enzyme. It is possible, therefore, that the protein-bound auxin may be the functional rather than the precursor or reserve form of auxin.

Ether-extractable and diffusible auxin, according to this view, could be a transport form of auxin produced from tryptophan but not yet combined with protein to form an enzyme. The relations between the various forms of auxin suggested here are summarized in Fig. 3.

#### ACKNOWLEDGMENTS

We are indebted to Dr. David Bonner of Yale University for the IPA and to Mr. F. W. Mitchell of this Institute for the indoleacetaldehyde used in these experiments. We are also indebted to Mr. Victor Schocken for his help on some of these experi-

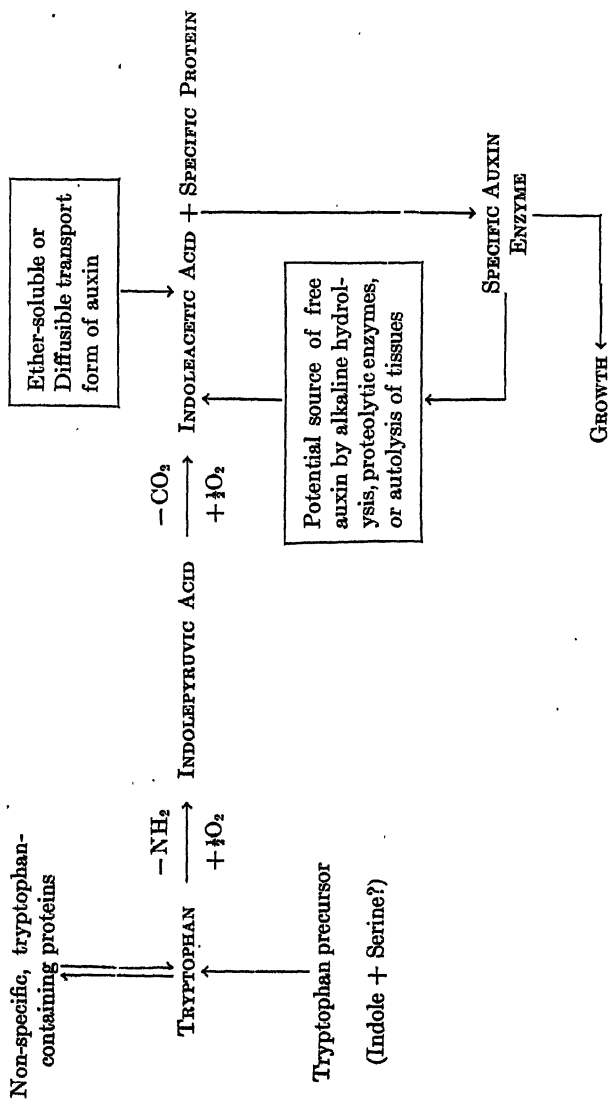


Fig. 3. A Schematic Representation of Possible Interrelations in Auxin Formation and Auxin Action.

ments. One of us (M. G. F.) wishes to express his gratitude to the Rockefeller Foundation for a fellowship which made this work possible. The microanalysis was made by Dr. G. Oppenheimer through the courtesy of Dr. A. J. Haagen-Smit, of this Institute.

### SUMMARY

1. When tryptophan is infiltrated into living spinach leaves, the ether-extractable auxin is increased 10 times in 3.5 hours over controls not treated with tryptophan.

2. Lyophilized, cell-free spinach leaf extracts rapidly convert tryptophan to auxin. The reaction is shown to be enzymatic.

3. A study of the mechanism of tryptophan conversion to auxin suggests that oxygen is required and that an intermediate compound containing a carbonyl group is formed during the conversion. Evidence is presented that indolepyruvic acid may be the keto intermediate formed and that indoleacetic acid may be the final product.

4. These experiments suggest that tryptophan is a principal auxin precursor in spinach leaves and that indoleacetic acid is a principal auxin.

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## LETTER TO THE EDITORS

### Interaction between Tobacco Mosaic Virus and Bovine Serum Albumin<sup>1,2</sup>

Kleczkowski (1) has recently reported the precipitation of tobacco mosaic virus (TMV) protein by various other proteins in the pH range between the isoelectric points of the virus and the precipitant. He showed that the precipitate consisted of mesomorphic fibers. Some other proteins were also found to form precipitates when mixed at pH values intermediate between their isoelectric points. These studies were carried out at extremely low ionic strengths. When sodium chloride was added, the precipitates dispersed.

In experiments soon to be published in detail, we found that TMV forms precipitates with bovine serum albumin when the pH of the medium is intermediate between the two isoelectric points and when the ionic strength is very low. Just as in the case reported by Kleczkowski, the addition of sodium chloride disperses the precipitate. However, we have observed the more interesting phenomenon that TMV protein is precipitated in the form of mesomorphic fibers by serum albumin at pH values alkaline to the isoelectric points of both proteins and also at pH values acidic to the isoelectric points of both proteins. In this case the precipitation is enhanced by the addition of electrolyte. It was found that the product of the electrolyte concentration and the serum albumin concentration required to just precipitate the virus was approximately a constant for low values of both the serum albumin and electrolyte concentrations. It was also found that the amount of serum albumin and electrolyte required to precipitate the virus was less at pH 5.8 than at pH 5.2. Chemical analyses seemed to indicate that the precipitate was composed largely of virus protein and little, if at all, of serum albumin.

Cohen (2) had previously observed that negatively charged hy-

<sup>1</sup> Contribution No. 11-47 of the Department of Physics of the University of Pittsburgh.

<sup>2</sup> Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

drophilic colloids, such as heparin, starch, gelatin, *etc.*, could be used to crystallize neutral solutions of TMV and other viruses. Stenhagen and Teorell (3) found evidence by means of electrophoresis of interaction between nucleic acid and human serum albumin at pII values at which both had negative charges. The results obtained in the present study throw further light upon the general question of the interaction between like charged colloidal particles. The interaction was shown to occur when both charges are positive as well as when both are negative. Neutral electrolytes were found to augment the interaction, and the extent of the interaction was shown to depend upon the magnitude of the charge of the particles involved. It would seem that the virus is literally pushed out of solution by the colloidal particles of like charge. These particles must have greater affinity for the solvent than the virus has. It is suggested that the neutral electrolytes act by decreasing the repulsion between virus particles more than the repulsion between virus and serum albumin.

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*Department of Physics,  
University of Pittsburgh,  
Pittsburgh 13, Pa.*

MAX A. LAUFFER

*February 14, 1947.*

## Book Reviews

**Lehrbuch der Physiologischen Chemie.** By EMIL ABDERHALDEN, Professor an der Universitaet Zürich. 23. bis 25., neu bearbeitete, erweiterte Auflage. Benno Schwabe und Co., Verlag, Basel, 1946. xii and 417 pp. Price: Fr. 26. (Swiss).

This textbook by the indefatigable editor of the well known biochemical and biological handbooks gives a very readable review of the field in 29 lectures. In its organization of the material it differs somewhat from most of the American textbooks on this subject. After an excellent introductory chapter of a general nature, nutrition in general and organic foodstuffs in particular are discussed. The chemistry of each group of substances, such as, *e.g.*, fats, phosphatides, steroids, carbohydrates, proteins *etc.*, is dealt with, together with their metabolism. These sections include some substances of a related chemical structure, but of a highly specific or even unrelated functional significance, such as some of the vitamins and hormones. The next chapters are concerned with blood and bile pigments, the vitamins and hormones not previously mentioned, and with enzymes. Sections on inorganic foodstuffs and other aspects of nutrition follow. The final chapters deal with gas and energy metabolism. The reviewer wonders whether an arrangement of some of the material more in line with the usual physiological classification (*e.g.* blood, urine, respiration, digestion, intermediary metabolism, vitamins, hormones, *etc.*) would not be preferable. It would avoid a scattering of some of these subjects throughout the entire book.

The book is very well written, and the subject matter made clear and easily understandable. Emphasis is put on fundamental and important concepts rather than on detailed facts and figures. The overall picture is always stressed and general interrelationships are never lost sight of. Along these lines may be mentioned as particularly valuable, *e.g.*, the general remarks made in the first chapter on physiological chemistry as related to other fields, on methods of research, on the significance of *in vitro* experiments as compared to investigations on living animals, and on critical interpretation of experimental results.

This emphasis on basic principles, found throughout the entire book, seems highly commendable, even if the author perhaps goes a little too far in this direction. Almost no tables are presented in the text, except in the last two chapters, and very few, if any, actual values are given for items such as composition of food, body fluids or tissues, not to mention physical or chemical characteristics of substances of biochemical importance.

As a whole the material is treated very uniformly. That some of the more recent developments, in particular those originating in countries outside of Central Europe, received a somewhat sketchy treatment, is understandable. The discussion of immunochemical problems is limited almost exclusively to a description of the "Abwehrfermente." In the opinion of the reviewer, it might have been desirable to also give some space to other aspects of immunochemistry, such as antigens and antibodies in general, antitoxins, and blood groups, to mention only a few.

The very extensive representation of chemical formulas and reactions is excellent. The illustrations likewise are very good, even if, in many instances, the source of origin is not given. The reviewer was unable to discover a single diagram in the whole book, and he wonders whether diagrammatic illustrations of principles such as are associated, *e.g.*, with buffers, indicators, solubility, pH optimum of enzyme activity, glucose tolerance, *etc.*, would not have aided the student appreciably in his efforts to understand and to remember.

Throughout the entire text, not a single name of an investigator is quoted. Credit is given only at the end of the book in a separate section which contains a sort of brief historical review for each chapter. Dr. Abderhalden explains in the preface that in previous editions it was not possible, for political reasons, to mention the names of various important investigators, and that he considered it fairer, at the time, to omit quotations altogether. This attitude appears highly commendable, as far as former editions are concerned which were published in Germany under the Nazi regime. It does not apply, however, to the present edition, and, in the opinion of the reviewer, it would have been preferable to incorporate names of investigators in the text.

The number of footnotes is very large, in many instances, important facts or concepts of a certain section are found in footnotes. This unnecessarily interrupts the flow of reading.

Paper and printing are excellent and the attractive binding compares favorably with that of most American books of this type.

The aforementioned points of minor criticism are more than compensated for by the excellent treatment of the fundamental principles and interrelationships. As a whole, the book is a valuable introduction into the field and can be highly recommended. It will be eagerly welcomed in Germany, where new editions of this sort are scarce or not available at all.

WALTER MARX, Los Angeles, Calif.

**Vitamins and Hormones, Vol. IV.** Edited by: ROBERT S. HARRIS, Professor of Biochemistry of Nutrition, Massachusetts Institute of Technology, and KENNETH V. THIMANN, Associate Professor of Plant Physiology, Harvard University. Academic Press, Inc., New York, N. Y., 1946. xvii + 406 pp. Price \$6.80.

This volume contains nine reviews of current topics. To generalize regarding the characteristics of the reviews as a whole or to evaluate them as a group would be difficult because of the diversity of the subjects treated and consequent differences in approach. In the following paragraphs an attempt will be made to indicate something as to the usefulness of each contribution.

The Newer Hematopoietic Factors of the Vitamin B-Complex, by J. J. Pffiffer and Albert G. Hogan (34 pages). Through no fault of the editors or authors the timing of this review was somewhat unfortunate because the crucial findings of the Lederle Laboratories regarding the structure of folic acid (pteroylglutamic acid) and related compounds had to be placed as an addendum. The clarifying effect of these findings could not be utilized in the main discussion which therefore had to deal to an unfortunate degree with numerous confused "factors", the names of which were often more distinctive than were the principles concerned. Judged on the basis of the situation which

existed when the review was written, it is sane, trustworthy and satisfactory. It is possible that the importance of microbiological work in this field is somewhat underrated and that not enough distinction was made between crude "factors", the existence of which as entities was postulated, and biochemical principles which were actually concentrated to a high degree.

**Nutrition and Resistance to Infection: The Strategic Situation**, by Howard A. Schneider (35 pages). This is a critical and careful review and is valuable whether or not one agrees entirely with the author's conclusions. He emphasizes the possible importance and relative lack of knowledge concerning the genetic constitution of the host as it affects resistance to infection and the fact that various nutritional factors may operate to increase either susceptibility or resistance to infection. He believes that the primary nutritional factors which are "profound and direct in their influence are yet to be isolated from natural products."

**Manifestations of Nutritional Deficiency in Infants**, by F. W. Clements (63 pages). This review with 437 literature citations contains a wealth of detail regarding 13 different types of nutritional deficiency, including undernutrition and lack of adequate water supply. Included is a considerable amount of elementary information which should be of value especially to clinicians who have not had adequate opportunity to keep abreast of nutritional advances. In view of the nature of the available data, the approach cannot be highly critical. Specific clinical manifestations are often ascribed to particular deficiencies with an assurance that the facts seem not to warrant. Deficiencies in human beings are more often than not multiple in nature and their identities have not been established with the certainty which results from controlled animal experiments.

**Effect of B Vitamins on the Endocrinological Aspects of Reproduction**, by Roy Hertz (12 pages). This short review deals with a field which is certain to develop in the years to come. Among the items stressed are: the indispensability of folic acid for estrogen response to stilbestrol in chicks, the pronounced but confused effects of B vitamins on lactation, and the relation of B vitamins in the maternal diet to deformities and death in the offspring and to hatchability of eggs. In this latter connection the striking work of Taylor on the effect of high levels of pantothenic acid on reproduction in fowls, rats and mice was not mentioned.

**Nutritional Therapy of Endocrine Disturbances**, by Morton S. Biskind (39 pages). Part of this review, that dealing with the importance of B vitamins in connection with promoting estrogen inactivation in the liver, is closely related to the preceding one. Other topics treated, for the most part critically and ably, include infertility, thyroid disturbances and diabetes. The authors of both this review and the preceding one emphasize the need for further extensive investigation.

**The Thyroid and Diabetes**, by Bernardo A. Houssay (20 pages). Factual material regarding experimental work dealing with numerous relations between thyroid functioning and intestinal absorption, glucose tolerance, glycogenolysis, sensitivity to adrenaline, pancreatic functioning, insulin action and sensitivity makes up the major part of this chapter. This is a field in which different species show wide differences and the results obtained with different animals and with human beings are properly distinguished and set apart.



**Thyroactive Iodinated Proteins**, by E. P. Reineke (48 pages). This well organized review gives in a comprehensive and satisfactory fashion the history and status of the iodination of proteins and the production thereby of thyroactive proteins and of thyroxin. The favorable effects of such iodinated proteins on milk secretion, body growth, feather growth and egg production are depicted.

**The Protein Anabolic Effects of Steroid Hormones**, by Charles D. Kochakian (56 pages). A large portion of this review has to do with the fundamental observation that administration of androgens causes increased nitrogen retention and decreased urinary nitrogen excretion. Detailed information is given regarding the effects of various natural and synthetic steroid hormones on urinary nitrogen excretion (qualitative as well as quantitative), on electrolyte balance, water metabolism, energy metabolism and tissue formation.

**Methods of Bioassay of Animal Hormones**, by Sidney A. Thayer (52 pages). In addition to reviewing the assay methods for the gonadotropic, adrenotropic, thyrotropic, lactogenic, and growth hormones of the pituitary, and likewise the various means of measuring the activity of adrenal cortical hormones, the author gives a good elementary discussion of the principles involved in such tests, the importance of animal variation and the statistical analysis of results.

ROGER J. WILLIAMS, Austin, Texas.

**The Chemistry of Heterocyclic Compounds**. By AVERY A. MORTON, Professor of Organic Chemistry in the Massachusetts Institute of Technology. McGraw-Hill Book Company, Inc., New York, N. Y., 1946. vii + 549 pp. Price \$6.00.

For many years a serious gap in the literature of organic chemistry has existed in the lack of an adequate modern and reasonably definitive treatment of the chemistry of the heterocycles. Professor Morton's book represents an effort to satisfy this need. As the author points out in the preface, the problem of selecting the essentials of this vast field and compressing them into a single volume of reasonable size is a formidable one. Any selection of material which is made must almost unavoidably run against individual views of readers of such a book and the present case offers no exception.

Perhaps the most novel feature of Professor Morton's treatise is his praiseworthy attempt to economize on space by use of partial structural formulas in which the major portion of such a formula is denoted by a large letter R with functional groups, generally those involved in the heterocyclic portion of the formula, emanating from the R. Likewise, rather than listing reagents conventionally on the reaction arrows, such notations have been accomplished by use of footnotes appearing at the bottom of a group of formulas. To this reviewer both of these novelties appear to be most unfortunate experiments, particularly the former. It is extremely difficult to form a visual image of a given structure expressed in this fashion without actually writing the complete formula.

Throughout the book a vast amount of detailed data, such as physical constants, *etc.*, on many substances discussed as well as on series of substances has been included. This has resulted in the utilization of much space which could have been utilized more profitably in incorporating additional facts dealing with chemical principles.

The grouping together of the azoles into one chapter on the basis of modes of formation and reactions represents a worthy effort at simplification in the classifica-

tion of heterocyclic compounds. One wonders whether simplification to such an extent is justified. A more logical arrangement would appear to be based on a subdivision on the basis of classification of such compounds as cyclic hydrazines, amidines, urcas, *etc.* As a result of the classification scheme used, to which has been added a discussion of resonance in such systems, certain unfortunate statements appear. For example, on p. 406 it is stated that resistance to the action of alkali in the case of azoles would depend on the degree to which resonance stabilizes the ring. On p. 402 the inertness of imidazole to substitution is attributed to resonance. But on p. 408 the statement appears that "benzoyl chloride and alkali cleave azoles smoothly, particularly in the case of imidazoles." What has happened to the resonance stabilization in this case? Other indiscriminate uses of the resonance concept appear throughout the book.

The treatment accorded the important reactions involving the reactivity of halogens and the hydrogens of methyl groups in the halogenated pyridines and the picolines, respectively, as well as in other similar heterocyclic types, leaves much to be desired. No discussion of modern electronic concepts as applied to the chloropyridines is given and the question of the reactivity of the hydrogens in the 2- and 4-picolines, quinolines, *etc.*, with the explanation thereof has merited no treatment at all. Likewise, the discussion of the alkyl pyridinium bases neglects the role played by the methylene bases in the chemistry of these important compounds.

It is to be regretted that the interesting investigations of the reactions by which furan derivatives are formed from aroyl ethylenes have not been accorded a more prominent place in the discussion of furan.

Throughout the volume this reviewer has found many evidences of careless assembling of the material such as incorrectly cited references both as to journal, location in the journal, year and volume. Many key recent papers have not been cited, *e.g.*, some of the most recent and accessible procedures for the nitration of quinoline and for the preparation of thiophene from acetylene, as well as several other very useful summarizing papers dealing with particular groups of substances. More serious are the numerous incorrect or misleading statements of fact which militate against the usefulness of the book except in the hands of a person thoroughly familiar with the field of chemistry under discussion.

A large number of, for the most part, well chosen problems appears at the ends of the various chapters. These have proved of help in teaching the subject to classes of graduate students.

ROBERT C. ELDERFIELD, New York, N. Y.

**Biology of Tissue Cells. Essays.** By ALBERT FISCHER, Head of the Biological Institute, Carlsberg Foundation, Copenhagen, Denmark. Stechert & Co., New York, N. Y., 1946. viii + 348 pp. Price \$6.50.

This is a work full of suggestions for intriguing and important research for the biochemist or physiologist interested in the fundamental aspects of his science.

It comprises twelve essays which, as the author states, "deal with problems within the general physiology and biology of tissue cells in their relation to organisms." The first two present the author's interpretation of the cell problem as it applies to the tissue cell in its relation to the organism and, in general, the modification of these relations in the tissue culture. He sees a clear dividing line in the integrated behavior,

modified, but not eliminated in culture, of the tissue cell and of the free living cell and asserts that the view that a culture of tissue cells and a culture of protozoa are directly and freely comparable is faulty. In this he lays great stress on the cohesiveness of the tissue cell and presents much evidence, mainly, however, of a qualitative and subjective nature, for the actual fusion by means of plasmodesma of the cell mass. Admitting that such connections are broken at times, he backs his contention as to the reality and physiological importance of such connection by citing the failure of all attempts hitherto to grow cultures from single cells. The obvious objection to this is that the present knowledge of the nutritional and environmental requirements of the individual cell is so meager that such failure is at present probably to be expected. For there can be no question that the cells modify the medium in their vicinity and probably must do so to grow. Their ability to effectuate a beneficial change would certainly be a function and probably not a direct function of their number.

It is certainly no criticism of these essays to raise objections to the interpretations made but merely proof of their stimulating quality. At each page the reader is confronted explicitly or implicitly with problems still open and requiring further research.

The next two essays are beautifully and thoroughly descriptive of the morphology of the cells in tissue culture. One point that has frequently been a source of argument between the pathologist or histologist and the worker with tissue culture is well discussed here. This is the use of the term "fibroblast" by the tissue culture worker to designate a group of cells of much wider range than the pathologist is willing to concede. It is stressed that for both the term is used to designate cells having the shape of the connective tissue cell as seen in fixed tissue. The difference in use arises because *in culture* the assumption of the morphologic characteristics so well known to the worker with fixed material are assumed by many cells which in fixed adult tissues do not appear to take on such morphology. They, as the author points out later, must be classified by their physiological (chemical) characters when in culture. Their morphology ceases to be a reliable criterion of origin. Much argument might be saved by wider recognition of these differences.

On the question of dedifferentiation the evidence presented as to the separability of morphology and function (as shown by the dedifferentiation of form which occurs widely, but leaves physiological response largely intact) is of great weight but argument here will doubtless still be carried on as before. It will not be settled conclusively until the culture from the single cell, or at least an extremely low number of cells, is achieved.

There is no doubt left in the readers' mind, however, after perusal and study of the material presented here, of the labile morphology encountered in tissue culture. It should be required study for all who habitually work with fixed tissues.

The next four essays, Rate of Growth, Tissue Culture as a Whole, Regeneration and Differentiation, and Organization, deal essentially with the differences and resemblances of the culture, taken as the unit of discussion, as contrasted with a complete organism. It is here that the weight of long experience and diligent experimentation by a master in the field tells most effectively. The discussion is closely reasoned, penetrating, and thorough.

The extent to which the culture behaves as a unit, *i.e.*, as an organoid, the relations found between the central portions of condensed, mechanically restrained tissue and

the active migratory proliferating fringe, and their concomitant physiological activity, the evidence presented for at least incipient "induction" as present in these "manufactured" organoid growths are fascinating, and arouse interest and posit problem after problem to the reader. There is little of the didactic in the account. Much of the interpretation is speculative but never strays far from observation.

The lack of quantitative expression is noticeable, but this is due to the inherent difficulties which go with any attempt at analysis of such complex phenomena and the time-consuming and costly techniques of culture, which make the control and frequent repetition necessary for obtainance of quantitative data in this field a herculean task.

The description of the phenomena of regeneration is very complete, but consideration of the interpretations advanced must always include the proviso on the part of the reader that it is speculative. The author rules out as primary factors the production of both growth-promoting and -inhibiting substances *per se* in determining both the initiation of growth after injury and its cessation at repair, but asserts that the "barrier" effect of the "dissociated" cells at the periphery of the culture is, together with a "concentration gradient," the dominant factor in producing cessation of growth on healing and its interruption by operation (excision) as initiating the outgrowth in regeneration. It must be noted that the nature of this "barrier" and gradient is an hypothesis *ad hoc* and not chemically defined. There is strong evidence presented for these hypotheses, but they await proof which it seems must be furnished by the biochemist.

The next four essays are devoted to the chemical aspects of the tissue cell in culture. There is an excellent critique and account of the classic attempt of Baker and Carrell to define the nature of the growth-stimulating substances for which evidence is strong. To this is added further work by the author along similar lines. His conclusions that these are in the nature of enzymes or dependent on unknown enzymatic actions are dependent on analogy in large part, always a weak position, and the tabulation of the stimulation given by various fractions of the nucleoproteins apparently largely responsible for the action do not present any clear correlation. Much more work of a difficult nature by the biochemist is evidently called for before much progress can be made. This is indeed indicated by the author's frank statement in the concluding sentence of the essay on growth-promoting substances that "what has been presented here is so far purely speculative."

The essay on nitrogen metabolism displays the ingenuity of the author in meeting the difficulties confronting the investigator in this field. The combination of barriers raised by the need for keeping the cultures and the materials sterile must have been great. This is not stressed but is evident to all with more than a reading acquaintance with the field.

The most important results are the evidence presented that the cells must be furnished with the higher split products of the proteins in order to utilize the amino acids in protein synthesis. Also the apparent inability of these cells to utilize methionine and the consequent essentiality of cystine.

In respect to the first point, one is reminded of the finding by Maver that increase in protein nitrogen obtained from hydrolyzates of proteins by oxygenation of solutions

containing high concentrations of the hydrolyzates and glutathione could not be obtained when the hydrolysis of the proteins was carried beyond 20%.

The experiments described would seem to furnish a basis for a real attack on the nutritional problem as respects the cell.

The discussion of the energy relationships points out the sharp resemblance of the oxidative metabolism of the culture to that of malignant tissue. It would be interesting to have studies of the *p*-phenylenediamine reaction here. The evidence for the utilization of glucose in growth is weighty but lacks the crucial proof that would have been given if dry weight could have been measured.

The concluding essay discusses the prospects for tissue culture. It hardly emphasizes sufficiently the difficulty of the technique involved. The real contributions are well brought out and it stresses again the author's concept of the organoid character of the culture and dependence of the individual cell upon this. Time, further research under more varied conditions, and improvement of techniques will finally confirm or refute these contentions.

It is a book for the advanced student, one for the reference shelf, and certainly, from the conceptual side, a fine summary of the researches in this field as evaluated by one who is the outstanding leader in the use of the tissue culture as a physiological tool.

As to the make-up of the book itself, it is greatly to be regretted that a far wider index was not provided, as it is quite inadequate.

There are too many typographical errors. Perhaps this is due to the aftermath of war.

The translator, Mr. Einar Christensen, has done, on the whole, an adequate piece of work. There are a good many "un-English" expressions and usages, but they do not interfere with readability.

HAROLD W. CHALKLEY, Bethesda, Md.

**Modern Developments in Chemotherapy**, from the series Research in Holland. By E. HAVINGA, H. W. JULIUS, H. VELDSTRADA and K. C. WINKLER. Elsevier Publishing Co., Inc., New York, Amsterdam, 1946. xi + 175 pp. Price \$3.50.

This modest but substantial and carefully prepared volume describing research in the field of chemotherapy conducted in Holland during the war years is one of many attestations of the remarkable record of accomplishment by scientists of the Netherlands during the five years of German occupation. The monograph, largely written while the war was still in progress, presents a summary of the results obtained, together with commentaries by leading chemists and bacteriologists associated with the investigations. The four principal authors are to be congratulated for having achieved a unified presentation of the work done and of the ideas and conclusions of the Netherlands' scientists.

The major part of the book is concerned with the problem of the mode of action of the sulfa drugs and of the antagonism by *p*-aminobenzoic acid. The problem was attacked on the one hand by bacteriological investigations (Julius, Winkler). For example, sulfanilamide was found to have no effect on bacterial respiration, amino acid synthesis, or on transamination; the action of sulfanilamide on bacteria in vitro is characterized by a growth curve of peculiar and suggestive form; a substance

present in horse blood is capable of enhancing the activity of sulfanilamide against various bacteria; the explanation of the antagonism of *p*-aminobenzoic acid for sulfanilamide in terms of the displacement theory is considered as probable but unproved. A second avenue of approach was by physico-chemical methods (Havinga, Veldstrada): electrometric titrations, determinations of ultraviolet absorption spectra, studies of the behavior of the substances in monolayers and at the mercury-solution boundary; a conclusion stressed by the authors is that the "surface activity" of the sulfanilamides is one of the factors determining their degree of chemotherapeutic activity. A third set of investigations was concerned with the synthesis of a number of additional heterocyclic variants of the sulfanilamide molecule.

The book is concluded with one short section on pharmacological and clinical investigations and another reporting a contribution to the knowledge of the use of antibiotic substances in combating pathogenic bacteria; the latter is concerned chiefly with investigations of a substance expansine, considered to be identical with patuline (clavacine).

LOUIS F. FIESER, Cambridge, Mass.

**L'Évolution biochimique.** MARCEL FLORKIN, Professeur à l'Université de Liège. Editions Desoer, Liège et Masson et Cie, Paris, 1944. 210 pp.

The theory of evolution is the grand generalization derived from a tremendous number of observations on plants and animals, which have been made by investigators using their ordinary senses, chiefly that of sight. In other words, the concept of biological evolution emerged from studies on the morphology of both living and extinct organisms. Embryology, or the study of the evolution of individual living forms, also made significant contributions to the general theory and these, too, were within the natural limitations of the scope of the seeing eye. But the idea that morphology is only the outward expression of submicroscopic phenomena has been making strong inroads into scientific thinking, and with it, as an obvious corollary, the thought is gaining acceptance that evolution itself is a process far beyond the range which vision can penetrate. Evolution, then, must manifest itself within the molecules of developing matter. This fundamental idea, already carried successfully into the realm of chemical embryology, is poignantly expressed by the quotation embellishing the fly leaf of the book: "Our final theory of evolution will see it largely as a biochemical process" (Haldane).

Of course, the time is not yet ripe to restate the theory of evolution in biochemical terms because our knowledge is still very fragmentary. But in the literature on this subject Florkin's "L'Évolution biochimique" is without question a very timely and brilliant contribution. Within the limited space of some two hundred pages he has packed a tremendous array of factual material. Less than one-tenth of the space is given over to what may be considered as speculative discussion. Indeed, one might wonder if the author should have confined himself to brief comments hardly commensurate with the intrinsic importance of the subjects under consideration. But such criticism seems scarcely justifiable since one cannot fail to realize with the author how inadequate and fragmentary our present knowledge is. This is still the time for much and hard spade work and for the present it may be the better part of wisdom to hold speculation within bounds. The author has obviously set himself a

definite but limited goal, and the fascinating little book is good proof that he has succeeded in achieving his objective.

The book is divided into seven chapters. In the first two the evidence of a unified biochemical plan in nature, as well as of certain dissimilarities, is discussed; the third and fourth chapters deal with the development of some biochemical components and the direction of their evolution within the animal kingdom; the last chapter is devoted largely to more or less theoretical considerations. More than half the text is given over to the fifth and sixth chapters replete with informative matter. Chapter V deals with biochemical adaptations or correlations between anatomical, physiological and ecological characteristics and special biochemical findings. In a very real sense, chapter VI is the backbone of the whole text since the main object of the inquiry is to show that the entire ensemble of biochemical characteristics furnishes a basis for taxonomic classification of animal organisms.

Beginning with the broadest classification of animals into invertebrates and vertebrates, the biochemical basis for this is discussed. In both, the supporting structures are made up of an organic substance impregnated with a mixture of mineral salts, but among invertebrates the organic substance is represented by spongin, or chitin, *etc.*, whereas in the vertebrates the substance is a cartilaginous matter consisting of a protein and chondroitin. Furthermore, the formation and maintenance of this biochemical structure in vertebrates has become dependent upon a complex mechanism involving a phosphatase enzyme, parathyroid hormone and vitamin D. A specialized substance, keratin, has been evolved as an integumentary covering only among vertebrates.

Among invertebrates, as a general rule, hydrolytic enzymes are all mixed together in a single digestive fluid, whereas among vertebrate animals these enzymes are localized in an orderly manner in different parts of the digestive tract. But even among invertebrates, in the more advanced forms, there is already evidence of varying degrees of separation and localization of enzymes.

The secretion of bile acids and bile pigments is peculiarly characteristic of vertebrates. However, among invertebrates one finds poisons resembling bile acids in their pharmacological action, and taurine, which in higher vertebrates exists entirely as the conjugated taurocholic acid in the bile, occurs in the free state among molluscs and crustaceans. Likewise, bile pigments (biliverdin  $\rightarrow$  bilirubin), which are also typical vertebrate products formed from hemoglobin through an intermediate substance, pseudohemoglobin, which loses its iron, find their counterpart in protoporphyrin, a product of hemoglobin catabolism of invertebrates formed from an intermediate, hema<sup>tin</sup>, which also loses its iron.

Purine catabolism in the two main classes of animals follows distinct paths: either adenine and guanine are set free and oxidized to hypoxanthine and xanthine, or adenosine and guanosine are liberated, acted upon by specific desaminases and finally converted to the same end products. Mammals (and vertebrates in general) possess guanase and the two specific desaminases, but lack adenase; the invertebrates, on the other hand, possess adenase and guanase, but lack the desaminases.

It is generally recognized that arginine is a predominant component of invertebrates while creatine predominates in vertebrate animals and the substance methylguanidine, which is closely related to creatine, is characteristic for vertebrates only.

Distinct differentiation in the chemical structure of vertebrates and invertebrates is revealed also in the mineral composition. Thus, the  $\frac{Na + K}{Ca + Mg}$  ratio in the body fluids of invertebrate animals is characteristically low (1.3-6.2) whereas in vertebrate animals it is high (13.1-61.1). In other words, the body fluids of vertebrates contain about ten times as much (Na + K) in relation to (Ca + Mg) as is ordinarily found in the body fluids of invertebrates. In this connection an interesting observation is reported. If the heart of a snail is suspended in a salt solution with a  $\frac{Na + K}{Ca + Mg}$  ratio adjusted to that of vertebrates, an electrocardiogram is obtained resembling in all essential respects that of a vertebrate heart. Also, if a frog heart is suspended in a solution with a ratio adjusted to the invertebrate level (snail), its electrocardiogram assumes a simple form.

This analysis is carried beyond the main separation of invertebrate and vertebrate organisms. The biochemical differentiation is shown in a most interesting manner in regard to various suborders, but the limits of a review would not permit further details.

It is unfortunate that the author, who provided the book with a good index of authors' names and subjects discussed, also a very useful index of the different animals mentioned in the text, failed to include a list of publications referred to. Such a bibliographic list would be a distinct improvement.

This delightful little book should be required reading for every student of biology, whether his primary interest is morphological, physiological or biochemical.

S. MORGULIS, Omaha, Nebr.

**Bacterial Chemistry and Physiology** By JOHN ROGER PORTER, Associate Professor, Department of Bacteriology, College of Medicine, State University of Iowa. John Wiley and Sons, Inc., New York, 1946. 1073 pp. Price \$12.00.

This book, according to the preface, has been the outgrowth of lectures given in a bacteriology course with the subject matter chosen and arranged primarily to fit the requirements and interests of graduate students. It contains 10 chapters: (1) Some physicochemical properties of bacteria and their environment; (2) The growth and death of bacteria; (3) The effects of physical agents on bacteria; (4) The effects of chemical agents on bacteria; (5) The chemical composition of microorganisms; (6) Bacterial enzymes and bacterial respiration; (7) Bacterial nutrition; (8) Metabolism of carbon compounds by microorganisms; (9) Metabolism of nitrogen compounds by microorganisms; and (10) Microbial fermentations.

The material presented in these chapters has been written in a lucid manner. The treatment has been, in general, thorough, with extensive bibliographies appended at the end of each chapter. The book will be read with profit not only by the students to whom it is directed but also by all biologists interested in cellular activities.

In the next edition of this excellent book the first chapter could be improved. The definition of osmotic pressure, for example, is rather weak: "the diffusion pressure of a solvent diffusing through a semipermeable membrane." The soundest and most general interpretation of this phenomenon is obtained from the standpoint of "escaping tendency" of Lewis and Randall. If the author had followed this treatment



he would not have fallen victim to the fallacy on p. 10, where the osmotic pressure of a solution of egg albumin containing 22.66 g./100 cc. (0.0067 *M*) is compared to that of a 4 % solution (0.111 *M*) of saccharose with the conclusion that the osmotic pressure of albumin is too low. A more extended treatment of this phenomenon and its use for the determination of molecular weights of proteins would be advisable. On reviewing oxidation-reduction potentials it would also be advisable to discuss the problem of electron-by-electron oxidation, so extensively treated by Michaelis and so important in its applications to biology. In fact, the two bacterial pigments, pyocyanine and chlororaphine, are typical examples of step-by-step oxidation. Some of the space devoted to the quinhydrone electrode could be more profitably given to a more thorough treatment of the glass electrode. A valuable addition to the many useful tables contained in this chapter would be one giving the pK values of all the weak acids useful for the preparation of buffers.

Any discussion of the effects of chemical agents on bacteria should include the problem of penetration through the cell walls, as it is of great importance. The antibacterial action of mercury, the author says (p. 273) "can best be explained on the basis that it interferes with some essential cellular metabolite, such as certain R-SH compounds." It is now generally admitted that Hg, as well as other heavy metals (Pb, Cd, Bi, Sn, Zn, Ag) and arsenic, are all toxic because they inhibit enzymes requiring the presence of -SH groups for activity.

The problem of bacterial stains is treated too superficially. A discussion of the Gram-positive, Gram-negative, and acid-fast bacteria would be useful, as these three bacterial groups not only differ in their staining properties, but also exhibit striking differential susceptibilities to the different types of antiseptics and antibacterial agents. For example, it has been shown that there is a correlation between the ability to retain the Gram stain and the presence around the cell of a Mg-ribonucleate complex (Bartolomew and Umbreit, *J. Bact.* 48, 567 (1944); Henry and Stacey, *Nature* 151, 671 (1943)).

On p. 405, in a list of some of the enzymes which take part in carbohydrate metabolism, *hexokinase* has erroneously been called *phosphatase*. The problem of carbohydrate synthesis by microorganisms could have been treated in the light of recent findings which have led to the abandonment of the view of "the resynthesis of glycogen from lactic acid." The resynthesis of carbohydrates, an oxidative phenomenon, starts generally with the oxidation of pyruvic acid. In the chapter on the synthesis of fats, the recent advances made in our knowledge of the metabolism of fatty acids and the interrelation between fats and carbohydrates have not been presented. The scheme for fat synthesis presented on p. 420 seems to be the least probable of all. In fact, reaction III, decarboxylation of pyruvic acid to acetaldehyde and CO<sub>2</sub>, occurs only in yeast and in some plants. Moreover, the role of *P* is overlooked in the series of 9 steps from glucose to a higher fatty acid.

The chapter on the general properties of enzymes (p. 455) would profitably include modern concepts of enzyme chemistry based on Michaelis's suggestion of the formation of the reversible enzyme-substrate complex, and on the importance of the protein moiety of the enzyme system, the only portion of the system possessing specificity. The problem of enzyme inhibition (protein-, prosthetic group-, coenzyme-, substrate-inhibitors) could be treated more extensively because it is as enzyme inhibitors

that the antibiotics act. For example, sulfanilamide is a substrate inhibitor, as it interferes with the formation of folic acid from *p*-aminobenzoic acid which is the substrate. The sulfhydryl reagents, such as heavy metals, arsenicals, halogen organic compounds are protein inhibitors, quite specific when used in small concentrations, and not "non-specific" as the author calls them on p. 463. Fluoride, HCN, and diethyldithiocarbamate are prosthetic group inhibitors, as they combine with the Mg, Fe-porphyrin, and Cu prosthetic groups.

In the recent controversy over the term *respiration*, some distinguished biologists have championed the heresy of dividing it into "aerobic" and "anaerobic" respiration, terms which have been accepted by Dr. Porter on p. 542. Lavoisier, the father of metabolism, defined respiration as the process which consists in the utilization of oxygen and production of CO<sub>2</sub>. There is no satisfactory reason for changing this age-old definition, because the oxidative processes which occur in the absence of oxygen may well be designated *fermentations*. There are also a few incongruities in Table 12, p. 547, which gives some properties and a classification of oxidation enzymes. Thus, among *oxidases* which "presumably activate oxygen" are grouped ascorbic acid and polyphenol oxidases (which the author would logically call dehydrogenation of ascorbic acid and of polyphenols by copper as the dehydrogenating agent), and among *dehydrogenases* are grouped as *aerobic*, *D*-amino acid "dehydrogenase" where the mechanism of oxidation of *D*-amino acids does not differ at all from that of ascorbic acid, except that here the hydrogen acceptor is flavin. The adoption, in its general terms, of Warburg's basic principles stated in his article in *Ergeb. Enzymforsch.* VII, 210 (1938), would bring the author to a more logical classification. In the chapter on enzyme variation and adaptation in microorganisms the important papers of Beadle, Tatum, Lindegren and Spiegelman should be discussed, as their contributions on the role of genes on enzyme formation are fascinating.

In the study of the metabolism of monobasic saturated acids (p. 802) the papers of Lynen on oxidation of acetic acid by yeast (*Ann.* 552, 270 (1942); 554, 40 (1943)) should be discussed because they represent an interesting attempt to clarify the mechanism of oxidation of this important intermediate of the metabolism of carbohydrates and fats.

The chapters on bacterial nutrition, on metabolism of carbon compounds, on metabolism of nitrogen compounds, and on microbial fermentations are excellent; they contain a large number of useful tables, and are a source of a great deal of information.

Dr. Porter has written an excellent book, and must be congratulated for this tremendous task. The few comments made have been mostly requests for additions, which perhaps the author omitted for lack of space, as the book extends to more than 1000 pages.

E. S. GUZMAN BARRON, Chicago, Ill.



# A Manometric Method for the Determination of Peroxidase Activity. Application to an Investigation of the Peroxidase of Germinating Cottonseed

A. M. Altschul and M. L. Karon

*From the Southern Regional Research Laboratory<sup>1</sup> New Orleans, Louisiana*

Received February 10, 1947

## INTRODUCTION

Peroxidase activity is usually determined by the method of Wilstätter *et al.* (1, 2), or by some modification (3, 4) of this method.

Although considerable progress in this field has followed the use of the above mentioned methods, there are definite limits to the use of the purpurogallin number as a measure of peroxidase activity. In order to compare the catalytic activity of a peroxidase preparation on a variety of substrates, it is necessary to express this activity in terms which are independent of the substrate used. If peroxidase activity is expressed as a rate constant such a comparison is possible. It is also possible then to quantitatively compare the activity of peroxidase preparations with that of other enzymes.

Altschul, Abrams and Hogness (5) expressed cytochrome c peroxidase activity in terms of a first order rate constant based on the measurement of the rate of disappearance of reduced cytochrome c in the reaction mixture. Balls and Hale (6) measured peroxidase activity by determining the rate of disappearance of hydrogen peroxide in a reaction mixture consisting of buffer, enzyme, hydrogen peroxide, and pyrogallol. Under their experimental conditions, peroxidase activity was expressed in terms of a zero order rate constant.

The manometric method described here consists essentially of titrating the unreacted hydrogen peroxide with a very active catalase preparation.<sup>2</sup> Peroxidase, buffer, hydrogen peroxide, and pyrogallol

<sup>1</sup> One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

<sup>2</sup> While this manuscript was being prepared a similar method was reported by Randall (7).

are allowed to react in a suitable flask at constant temperature for 5 minutes, after which catalase is added and the amount of oxygen liberated is measured. A similar manometric method was employed by Elliott (8) with the addition of manganese dioxide as catalyst for the decomposition of the peroxide but it was found that the presence of various substances in the reaction mixture affected the catalytic decomposition of the peroxide.

### METHOD

The reaction was carried out in a Warburg constant volume manometric apparatus with reaction vessels having two side-arms. Buffer, pyrogallol, and the peroxidase solution were introduced into the main vessel, hydrogen peroxide solution into side-arm No. 1, and the catalase solution into side-arm No. 2. After equilibration in the bath at 30°C., the stopcock on the manometer was closed, hydrogen peroxide was tipped into the vessel, and the reaction allowed to continue for 5 minutes. At the end of the reaction period, catalase was tipped into the reaction mixture. Oxygen evolution began immediately and constant manometer readings were obtained within 10 minutes.

In a typical experiment the reacting system consisted of 2.0 ml. of *M*/30 phosphate buffer (pH 6.8), 0.15 ml. of 3% pyrogallol solution, 0.15 ml. of a 1/100 dilution of commercial 30% hydrogen peroxide, 0.40 ml. of peroxidase solution, and 0.30 ml. of catalase solution. The volume of oxygen evolved was 107.0 mm<sup>3</sup>. compared to 141.0 when peroxidase was absent from the mixture.

The rate constant for this reaction was calculated by means of the equation for a first order reaction:

$$k = \frac{2.303}{t} \log \frac{V_0}{V} \quad (1)$$

where  $k$  is the first order rate constant,  $t$  is the duration of the peroxidase reaction,  $V_0$  is the volume of oxygen released by catalase in the absence of peroxidase, and  $V$  is the volume of oxygen released after the peroxidase reaction had taken place for the length of time,  $t$ . The rate constant,  $k$ , calculated for the above-described test is 0.055.

### ENZYME PREPARATIONS

The peroxidase content of cottonseed increases upon germination. The extent of peroxidase formation cannot, however, be measured until the considerable quantity of catalase which is also present is first destroyed. Kasanski (11) reported that catalase activity can be selectively destroyed by incubating the enzyme solutions with pyrogallol. A modification of this procedure was applied to cottonseed after germination for 5 days at 25°C. The seedlings were extracted 3 times with an excess of cold acetone by agitation in a Waring Blender. The acetone-extracted residue was air-dried and ground to pass through a 60-mesh screen. One gram of the air-dried powder was suspended in 10 ml. of *M*/15 phosphate buffer of pH 7, and maintained at 3°C. over night. The mixture was centrifuged and the residue homogenized with more of the same buffer and again centrifuged. The residue was again extracted by a repeti-

tion of homogenization and centrifugation, and all the extracts were combined and diluted with buffer solution to give a total volume of 25 ml. Pyrogallol (1.25 g.) was added to the combined extract after which it was incubated at 3°C. for 24 hours. After incubation the solution was clarified by centrifugation and dialyzed against running distilled water for 24 hours at 3°C. The dialyzed solution was again clarified by centrifugation. It exhibited peroxidase but no catalase activity and was therefore used as the source of peroxidase.

Loew (9) reported that cottonseed meal is a good source of catalase and this observation has been confirmed by Olcott and Thornton (10). Catalase was prepared from cottonseed kernels which were extracted 3 successive times in a Waring Blendor with cold acetone. The meal was then dried at room temperature and ground to pass through a 60-mesh screen. A mixture consisting of 1 g. of this powder suspended in 5 ml. of *M*/15 phosphate buffer of pH 7 was used to liberate the unreacted hydrogen peroxide in the peroxidase test. This mixture does not contain any appreciable quantity of peroxidase.

### EXPERIMENTAL

*Order of Reaction.* Under the above-described experimental conditions, in which the initial hydrogen peroxide concentration is maintained constant, the activity of peroxidase can be expressed in terms of a first order reaction constant as is indicated in Table I. Within the

TABLE I  
*Determination of Order of Peroxidase Reaction*

Reaction time	Enzyme conc. <sup>a</sup>	Peroxide used <sup>b</sup>	Calculated rate constant (per ml. of enzyme/min.)	
			Zero order <sup>c</sup>	First order
<i>min.</i>	<i>ml.</i>	<i>per cent</i>		
6	0.4	60.6	27.4	0.39
4	0.4	47.0	31.7	0.37
3	0.4	39.8	35.8	0.42
6	0.2	38.4	38.2	0.40
4	0.2	25.9	38.8	0.38
3	0.2	21.6	43.0	0.40

<sup>a</sup> Refers to amount of peroxidase added in a total test volume of 3 ml. The other components of this test mixture were kept constant at 2 ml. *M*/30 phosphate buffer (pH of 6.8), 0.15 ml. of freshly prepared 3% pyrogallol solution, 0.15 ml. of freshly prepared hydrogen peroxide solution (commercial 30% hydrogen peroxide diluted 1/100), and 0.3 ml. of catalase preparation.

<sup>b</sup> Refers to fraction of total available hydrogen peroxide utilized in peroxidase catalyzed reaction.

$$^c k = \frac{C_0 - C}{t}$$

range of 20–60% utilization of the hydrogen peroxide, the rate constant calculated from Eq. (1) is constant, whereas that calculated from the equation for a zero order of reaction varied from 27.1 to 43.0.

*Peroxidase Concentration.* The data in Table I indicate that the rate constant is proportional to the enzyme concentration over a wide range of utilization of hydrogen peroxide. In another series of experiments in which the duration of the peroxidase reaction was kept constant at 5 minutes and the enzyme concentration was varied, the rate constant was also proportional to the enzyme concentration within the range of hydrogen peroxide utilization of 23–72% as indicated by Fig. 1, where the peroxidase concentration is plotted against the rate constant.

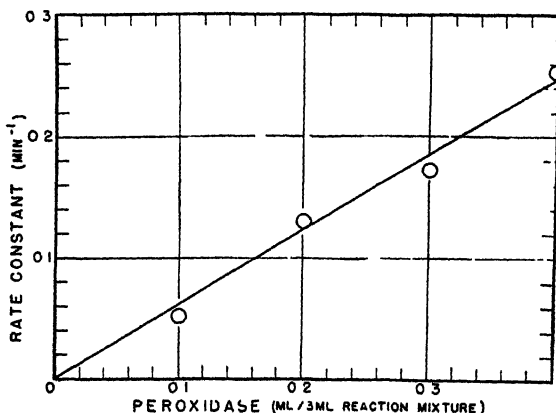


Fig. 1. Effect of Peroxidase Concentration on Value of Rate Constant. The fraction of hydrogen peroxide which was reduced in 5 minutes when the concentration of peroxidase was 0.1 and 0.4 ml./3 ml. was 23% and 72%, respectively.

*Hydrogen Peroxide.* Since the rate of reduction of hydrogen peroxide is proportional to its concentration, the rate constant can be calculated from the equation for a first order reaction. However, for any given enzyme concentration, the rate constant is influenced by the initial concentration of hydrogen peroxide. The reaction, therefore, is not truly first order with respect to the hydrogen peroxide concentration.

In a series of experiments in which the enzyme concentration was maintained constant and the initial concentration of hydrogen peroxide was varied it was found that the value of the rate constant decreased as shown in Fig. 2. This dependency of the rate constant on

initial concentration has previously been demonstrated by Willstätter and Weber (12) and Sumner and Gjessing (13).

The initial velocity of the reaction is obtained by multiplying the rate constant by the initial concentration of hydrogen peroxide. The curve showing the effect of initial hydrogen peroxide concentration on the initial velocity is also given in Fig. 2. The maximum initial velocity is obtained when a concentration of approximately 0.25 ml. of a 1/100 dilution of commercial 30% hydrogen peroxide per 3 ml. of test

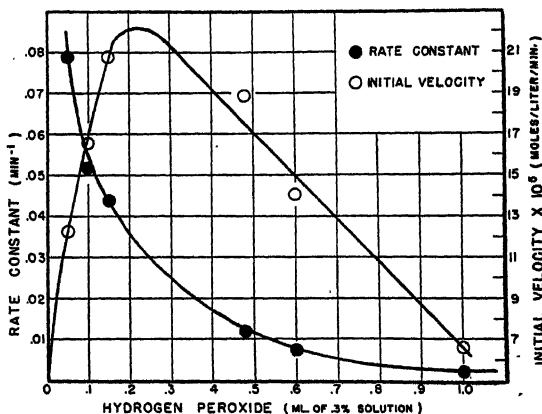


FIG. 2. Effect of Initial Hydrogen Peroxide Concentration on Rate Constant and Initial Velocity of Peroxidase Catalyzed Reaction. The other components of the test mixture were 2 ml. *M*/30 phosphate buffer (pH 6.8), 0.15 ml. of 3% pyrogallol solution, 0.40 ml. of peroxidase solution, and 0.80 ml. of catalase solution. Total volume was 3 ml.

sample ( $8 \times 10^{-3} M$ ) is used. It is clear that care must always be taken to use the same initial concentration of hydrogen peroxide in order to obtain concordant results.

*Pyrogallol.* It has been shown that pyrogallol inhibits catalase activity and in these experiments any inhibition due to excess pyrogallol would have resulted in an apparent increase in the peroxidase rate constant. It was, therefore, essential to use the lowest concentration of pyrogallol which would give uniform results.

To determine the effect of the concentration of pyrogallol on the rate constant, a series of experiments were made in which all of the reactants were maintained constant with the exception of the pyro-



gallol with the results which are shown graphically in Fig. 3. Reference to this figure shows that, with increasing pyrogallol concentration, the peroxidase rate constant increases even at rather high concentrations of pyrogallol. It is possible that the second part of the curve (above 0.3 ml. of pyrogallol) represents an apparent increase in the rate constant not as a result of the effect of pyrogallol on the peroxidase reaction itself but rather to the inhibition of catalase by the high concentrations of pyrogallol.

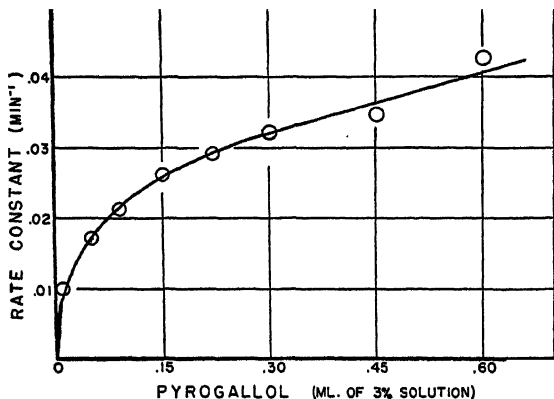


Fig. 3. Effect of Pyrogallol Concentration on Rate Constant of Peroxidase-Catalyzed Reaction. The other components of this test mixture were 2 ml. buffer, 0.15 ml. of a 1/100 dilution of commercial 30% hydrogen peroxide, 0.40 ml. of peroxidase solution, and 0.30 ml. of catalase solution.

That the activity of catalase is inhibited by high concentration of pyrogallol is evident from the fact that the amount of oxygen liberated from hydrogen peroxide is decreased with increasing concentration of pyrogallol as shown in Table II.

The concentration of pyrogallol used in these experiments was such that it yielded a relatively high peroxidase rate constant without seriously interfering with the action of the catalase.

*Catalase.* In a series of experiments using the concentrations of hydrogen peroxide and pyrogallol given in Table I and a constant amount of peroxidase, a peroxidase rate constant of 0.22/min. was obtained when 0.3 ml. and 0.1 ml. of the catalase suspension were used. A rate constant of 0.30 was obtained when only 0.05 ml. of catalase

TABLE II

*Effect of Pyrogallol Concentration on Volume of Oxygen Liberated from Hydrogen Peroxide by the Action of Catalase*

Pyrogallol <sup>a</sup> ml.	Oxygen liberated mm <sup>3</sup> .
0.01	155
0.015	152
0.05	153
0.09	146
0.15	141
0.22	137
0.30	136
0.45	120
0.60	116
1.8	16.5

<sup>a</sup> The concentration of pyrogallol is expressed in ml. of 3% solution/3 ml. of total reactants.

The commercial source of hydrogen peroxide used in these tests contained 30.9 g. of hydrogen peroxide/100 ml. of solution. The maximum volume of oxygen under standard conditions of temperature and pressure that could be obtained from the quantity of hydrogen peroxide used per test was, therefore, 153 mm<sup>3</sup>. From Table II it is seen that maximum amounts of oxygen were evolved with concentrations below 0.05 ml. of 3% pyrogallol solution in 3 ml. of test mixture. At higher concentrations of pyrogallol, the catalase was inactivated before all of the hydrogen peroxide was decomposed. When 0.45 ml. of pyrogallol solution was added, the decrease in total oxygen evolution became pronounced, and when 1.8 ml. was added, almost complete inhibition of catalase activity occurred as indicated by the small volume of oxygen evolved.

suspension was used. It is clear that the 0.05 ml. of catalase is insufficient and the 0.3 ml. of catalase more than sufficient to give a satisfactory peroxidase rate constant. An adequate factor of safety is, therefore, provided when 0.3 ml. of a catalase<sup>3</sup> preparation is used.

*Effect of pH.* The activity of peroxidase from cottonseed is quite sensitive to the pH of the reaction medium. In a series of experiments using a fixed peroxidase concentration and varying the pH of the test

<sup>3</sup> This catalase preparation had an activity,  $k$ , of 14 units/ml./min. when determined at 0°C. using a 0.0035  $M$  hydrogen peroxide concentration. The value of  $k$  is calculated by use of the following equation:

$$k = \frac{2.303}{t} \log \frac{C_0 - C}{C},$$

where  $C_0$  is the original concentration of hydrogen peroxide, and  $C$  is the concentration of peroxide after a period of reaction of  $t$  minutes.

mixture, the value of the rate constant was found to be 0.018/min. at pH 5.13, 0.041 at pH 5.90, and 0.078 at pH 6.95. The effect of pH in the alkaline range could not be determined with pyrogallol as the substrate because of the rapid chemical oxidation of this material at pH values above 7.

### KINETICS

Lineweaver and Burk (14) developed equations applicable to the determination of the enzyme dissociation constants for a number of enzyme-catalyzed reactions, one type of which is catalyzed by catalase and oxygenase. In this type of reaction, marked inhibition results from an excess of substrate. For example, the initial velocity of decomposition of hydrogen peroxide catalyzed by catalase increases with increasing peroxide concentration up to a maximum and then falls abruptly with further increase in concentration of the substrate. The curve relating the initial concentration of hydrogen peroxide to catalase activity indicates that the effect on the initial velocity of this reaction is similar to that observed with hydrogen peroxide on the peroxidase reaction shown in Fig. 2.

The above-mentioned authors consider the catalase reaction an example of the formation of two enzyme substrate complexes, one active and the other inactive, which may be expressed mathematically as follows:



where  $E$  represents the enzyme molecules,  $S$  the substrate molecules, and  $ES$  and  $ES_n$  the enzyme substrate complexes.

The velocity equation involved in substrate inhibition as given by Haldane (15) is:

$$v = V_{\max}[S]/([S] + K_s + [S]^n/K_2) \quad (4)$$

Where  $v$  is the initial velocity;  $V_{\max}$  is a numerical constant representing the maximum velocity obtained when the enzyme exists completely in the form  $ES$ ;  $K_s$  is the dissociation constant of the active enzyme substrate complex;  $K_2$  is the dissociation constant of the inactive enzyme substrate complex; and  $n$  is the number of moles of substrate combined/mole of enzyme in the inactive complex. For convenience in determining the values of the constants, Lineweaver and Burk expressed Eq. 4 in the following two forms:

$$[S]/v = K_s/V_{\max} + (1/V_{\max})([S] + [S]^n/K_2), \quad (5)$$

and its logarithmic form,

$$\log([S]/v - K_s/V_{\max} - [S]/V_{\max}) = n \log [S] - \log K_2 V_{\max}. \quad (6)$$

As pointed out by Lineweaver and Burk, for low values of  $[S]$ , Eq. (5) may be

reduced to the form:

$$[S]/v = K_s/V_{\max} + 1/V_{\max}[S]. \quad (7)$$

When the values of  $[S]/v$  of Table III are plotted against the corresponding values of  $[S]$  at low concentrations, a straight line is obtained, as shown in Fig. 4, whose slope is 3,000 and intercept at  $[S] = 0$  is 8.8. Substitution of these values in Eq. (7) gives a value of  $V_{\max} = 3.33 \times 10^{-4}$  M/l./min. and of  $K_s = 2.94 \times 10^{-3}$  M/l.

TABLE III  
*Calculation of Values of  $[S]/v$  as a Function of the  
Concentration of Hydrogen Peroxide*

Hydrogen peroxide, <sup>a</sup> solution/3 ml.	Conc. of substrate [S]	Peroxidase rate constant	Initial velocity, <i>v</i>	[S]/ <i>v</i>
ml.	M/l.	<i>k</i>	M/l./min.	
0.05	$1.58 \times 10^{-3}$	0.079	$12.3 \times 10^{-5}$	12.9
0.10	$3.17 \times 10^{-3}$	0.052	$16.6 \times 10^{-5}$	19.1
0.15	$4.75 \times 10^{-3}$	0.044	$20.8 \times 10^{-5}$	22.8
0.48	$15.23 \times 10^{-3}$	0.012	$18.9 \times 10^{-5}$	80.7
0.60	$19.00 \times 10^{-3}$	0.0074	$14.1 \times 10^{-5}$	135.1
1.00	$31.7 \times 10^{-3}$	0.0021	$6.65 \times 10^{-5}$	476.2

<sup>a</sup> The commercial product used as a source of hydrogen peroxide contained 32.4 g. of peroxide/100 ml. of solution. The peroxide solution used in these experiments was a 1/100 dilution of this commercial product.

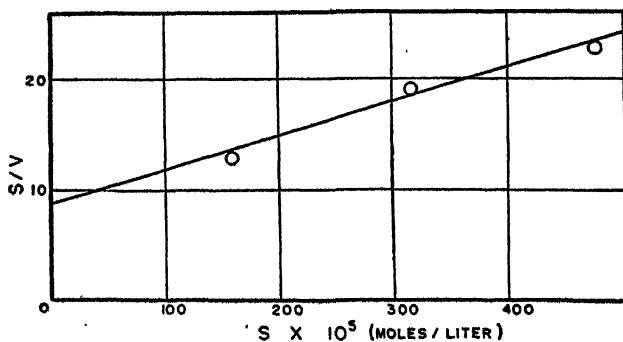
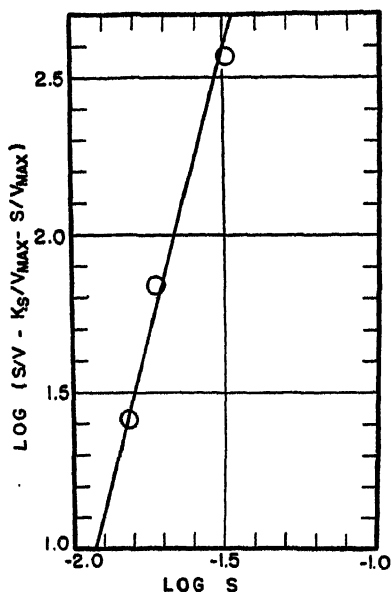


FIG. 4. Determination of Provisional Values of  $K_s$  and  $V_{\max}$ .

If values for  $\log ([S]/v - K_s/V_{\max} - [S]/V_{\max})$  are plotted against the logarithm of the corresponding values of  $[S]$  at high concentrations the values of  $n$  and  $K_2$  can be determined as shown in Fig. 5. The straight line thus obtained has a slope of 3.80, and an intercept at  $\log[S] = 0$  of 8.33, from which it is found that  $n = 4$  and  $K_2 = 1.41 \times 10^{-5}$  M/l.

FIG. 5. Determination of Value of  $K_2$  and  $n$ .

When the values for  $[S]/v$  were plotted against the corresponding values for  $[S] + [S]^n/K_2$  obtained by means of the numerical values of  $n$  and  $K_2$ , a straight line is obtained, as shown in Fig. 6, whose slope is 4,400 and intercept at  $[S] = 0$  is 5.5.

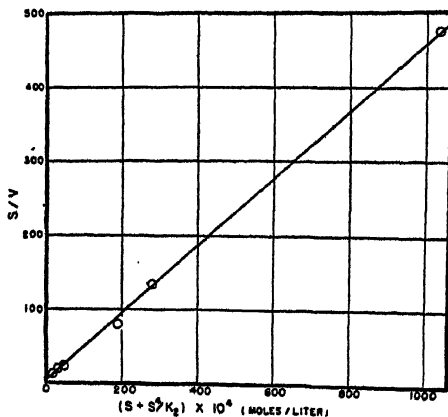


FIG. 6. Determination of the Validity of Eq. (6) over the Entire Range of Substrate Concentration.

When the various numerical values enumerated above are substituted in Eq. (4) the following series of values are obtained.

$$\begin{aligned}K_s &= 1.25 \times 10^{-3} \text{ M/l.}, \\V_{\max} &= 2.3 \times 10^{-4} \text{ M/l./min.}, \\n &= 4, \\K_2 &= 1.4 \times 10^{-5} \text{ M/l.}\end{aligned}$$

To check the validity of the above analysis of peroxidase kinetics, the initial velocity of the peroxidase reaction was calculated over a wide range of hydrogen peroxide concentration by substituting the values for the constants  $K_s$ ,  $V_{\max}$ ,  $n$ , and  $K_2$  in Eq. (4). From the values of initial velocity thus obtained, a theoretical curve relating initial velocity to initial hydrogen peroxide concentration was drawn as shown in Fig. 7. The smooth curve is the theoretical curve and the circles represent the experimentally determined values.

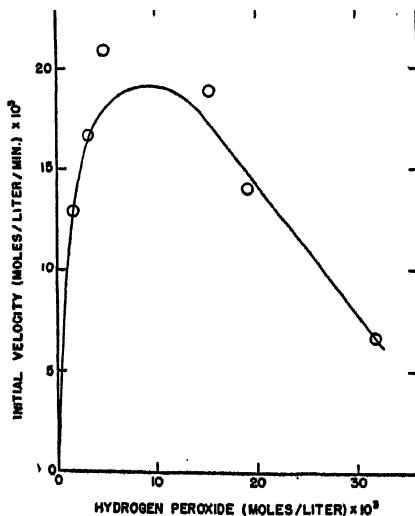


Fig. 7. Initial Velocity as a Function of Initial Hydrogen Peroxide Concentration. The curve represents calculated values while the circles represent experimentally determined values.

## DISCUSSION

The existence of active and inactive peroxidase-hydrogen peroxide complexes was originally proposed by Willstätter and Weber (12) and subsequently by Mann (16) to explain the reversible inhibition of

peroxidase activity by excess of peroxide. The fact that the analytical method of Lineweaver and Burk (14), which is also based on the assumption of such complexes (15), may be applied to the kinetics of cottonseed peroxidase activity lends further support to the validity of the foregoing hypothesis.

The values of  $K_s$  and  $K_2$  calculated for cottonseed peroxidase are valid only for the experimental conditions under which they were obtained. Mann (16) has shown that the concentration of reducing substrate affects the value of the Michaelis dissociation constant for the peroxidase-hydrogen peroxide complex. As the concentration of the reducing substrate is increased, the value of the dissociation constant also increases. When Mann used leuco malachite green in a concentration of 0.007% as the reducing substrate at a pH value of 4, he obtained a value for the dissociation constant of  $5 \times 10^{-6} M$ . When Chance (17) used the same reducing substrate in a concentration 0.1 that used by Mann and at the same pH value, he obtained a value for the dissociation constant of the peroxidase-peroxide complex of  $0.5 \times 10^{-6} M$ . Mann (16) also determined the effect of initial concentration of hydrogen peroxide on the velocity of peroxidase catalyzed oxidation of guaiacol. In this case the guaiacol concentrations were much greater than in the case of the leuco malachite green, and the Michaelis constants were correspondingly higher. For example, the Michaelis constant calculated from a guaiacol concentration of 0.125% at a pH value of 4.7 was found to be approximately  $8 \times 10^{-4} M$ .

Van Slyke (18) pointed out that the pH of the reaction medium may greatly influence the dissociation of the enzyme-substrate complex. There is, therefore, no *a priori* reason for comparing the results obtained by the action of cottonseed peroxidase on pyrogallol with those of Mann (16) or Chance (17) who used different reducing substrates at lower concentrations and in more acid media.

### SUMMARY

1. A manometric method for the determination of peroxidase activity has been described. The method consists of reacting hydrogen peroxide with a suitable reducing substrate in the presence of peroxidase for 5 minutes, after which the remaining unreacted peroxide is decomposed by means of catalase, and the amount of oxygen released is measured manometrically.

2. The method has been applied to an investigation of peroxidase from cottonseed, and the order of the reaction and effects of substrate concentration and pH have been determined.

3. The kinetics of the effect of initial hydrogen peroxide concentration on cottonseed peroxidase activity has been investigated, and evidence has been obtained to support the hypothesis that peroxidase forms both active and inactive complexes with hydrogen peroxide.

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# Interaction of Calcium, Phosphorus and Vitamin D.

## I. Influence of Dietary Calcium and Phosphorus on Body Weight and Bone Ash of Chicks\*

B. B. Migicovsky<sup>1</sup> and A. R. G. Emslie<sup>2</sup>

*From the Vitamin and Physiological Research Lab.<sup>3</sup>,  
Experimental Farm, Ottawa, Canada*

Received November 20, 1946

### INTRODUCTION

It is common knowledge that the calcium and phosphorus content of the rachitogenic diet has a marked influence on the response to vitamin D. Studies of this nature have been made on rats by Brown *et al.* (1932), Bethke *et al.* (1932), Booth *et al.* (1942) and Schneider and Steenbock (1939). The effect of calcium and phosphorus levels and ratio on growth and calcification in the chick has been demonstrated by Bethke *et al.* (1929) and Hart *et al.* (1929). McGowan and Emslie (1934) accentuated the fact that there are two types of "rachitic" lesions, one is associated with a high calcium diet, the other with a low calcium diet. Jones (1946) found that the slope of a dose response curve changes with the calcium-phosphorus ratio.

These initial experiments were conducted to confirm the finding of the above investigations, and to extend the range of the calcium and phosphorus content in the diet.

All trials were conducted with day-old white leghorn chicks, which were kindly furnished by the Poultry Division of the Central Experimental Farm, Ottawa.

The basal diets used in this investigation were the A.O.A.C. (1940) vitamin D chick assay diet and a modification of it, the composition of which is shown in Table I. In diets varying in calcium and phosphorus content the levels of calcium and phosphorus were attained by altering the amounts of  $\text{CaCO}_3$  and  $\text{KH}_2\text{PO}_4$  at the expense of ground yellow corn and  $\text{Ca}_3(\text{PO}_4)_2$ . Experiments 61, 69 and 71 were carried out with A.O.A.C. basal diet and 104 and 105 with the modified basal diet.

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\* Scientific Contribution No. 127 from the Division of Chemistry, Science Service.

<sup>1</sup> Associate Chemist, Production Service.

<sup>2</sup> Chemist, Science Service.

<sup>3</sup> The Vitamin and Physiological Research Laboratory was established on a collaborative basis by Experimental Farms, Production and Science Services of the Dept. of Agriculture, Canada.

TABLE I  
*Composition of Basal Diets*

	A.O.A.C. (1939)	Modified
Ground Yellow Corn	58	45
Ground Middlings	25	20
Casein	12	13
Buttermilk Powder		8
Yeast	2	3
Ceroglass		5
$\text{Ca}_3(\text{PO}_4)_2$	2	
$\text{CaCO}_3$		4
$\text{KH}_2\text{PO}_4$		1
$\text{NaCl}$	1	1
$\text{MnSO}_4$	9 g./100 lb.	9 g./100 lb.

The chicks were placed in battery brooders in groups of 15 to 20, and kept on experiment for three weeks. They were weighed at the end of the three week period, killed and the left tibiae removed. The bones were cleaned, extracted (alcohol followed by ether) and ashed as described in the A.O.A.C. (1940) chick assay for vitamin D.

The source of vitamin D was Canadian Standard Reference Oil No. 1 which has the same vitamin D activity as USP Reference No. 2. The reference oil was diluted with corn oil for the positive controls, and a total of 1% of oil was added to both positive and negative control diets. All diets were analyzed for calcium and phosphorus, using perchloric acid digestion followed, in the case of calcium, by permanganate titration and in the case of phosphorus by colorimetric determination. The phytin phosphorus content of the basal diets averaged 0.25% phosphorus. As this was relatively constant in all diets, and as no attempt was made to partition the phosphorus further, values are given for total phosphorus only.

## RESULTS

Random paired groups of chicks were fed diets which differed in calcium and phosphorus content. One group of each pair received no vitamin D and the other received 100 A.O.A.C. units/100 g. diet, thus enabling a comparison of chicks on diets of different calcium and phosphorus content, with and without vitamin D.

The values given in the subsequent tables for necessary difference for significance at  $P = 0.01$  were calculated from the variance within groups. The error is probably underestimated to some extent but we feel it is not sufficient to alter the observations noted. Actual values for bone ash and body weight obtained with different hatches have not been compared directly, but the relative changes which have resulted from increasing the calcium or phosphorus content of the diet, or from supplying vitamin D, have been compared. Our facilities did not per-

mit of replicating adequately at any one time all the diets being investigated.

The effect of increasing the calcium and phosphorus level and maintaining the ratio constant is shown in Table II.

TABLE II  
*Effect of Increasing the Dietary Level of Calcium and Phosphorus at a Constant Ratio of Calcium to Phosphorus*

Exp. No.	Ca	P	Ca/P	Bone ash		Body weight		Necessary difference at $P=0.01$	
				Negative control	Positive control	Negative control	Positive control	Bone ash	Body weight
	<i>per cent</i>	<i>per cent</i>		<i>per cent</i>	<i>per cent</i>	<i>g.</i>	<i>g.</i>	<i>per cent</i>	<i>g.</i>
61	1.16	0.39	3.0	29.88	44.48	130.9	154.3	1.94	15.7
	2.00	0.70	2.9	38.50	44.54	154.0	149.7		
61	0.72	0.37	1.9	25.71	44.22	115.5	166.8	2.01	16.2
	1.35	0.68	2.0	32.11	45.30	138.0	162.0		
71	1.07	0.51	2.1	34.57	44.80	133.0	160.0	2.14	18.9
	2.03	0.95	2.1	39.39	46.18	139.0	137.0		
71	0.63	0.52	1.2	29.01	44.68	134.0	161.0	2.24	18.9
	0.99	0.81	1.2	34.46	44.75	136.0	158.0		
104	2.83	0.56	5.05	33.11	40.96	110.0	95.0	2.13	12.8
	4.44	0.78	5.7	41.83	45.59	93.0	94.0		
104	0.56	0.74	0.76	28.31	41.90	92.0	133.0	2.07	12.5
	1.04	1.41	0.74	33.24	45.39	79.3	123.0		
105	1.05	0.58	1.83	33.71	43.06	128.5	130.1	2.06	17.1
	1.96	0.94	2.09	40.20	47.16	132.9	148.4		
106	0.55	0.94	0.59	28.52	43.08	83.3	147.9	2.27	18.9
	1.05	1.78	0.59	31.95	46.88	62.6	101.5		

At all Ca:P ratios the *per cent* bone ash of the negative control groups increased when the level of mineral in the diet was increased. There was no significant change in *per cent* bone ash of the positive control groups except at very low or very high ratios (Exp. 104 and 106) in which case the bone ash increased as the level of mineral was increased.

The body weight of the negative control groups did not change except when the initial phosphorus level was low (less than 0.5%). When the level of mineral was such that the Ca:P ratio was less than 1 or more than 4, the body weight decreased upon addition of mineral. The body weight of the positive control groups remained relatively unchanged with increase in mineral level. The difference in body weight

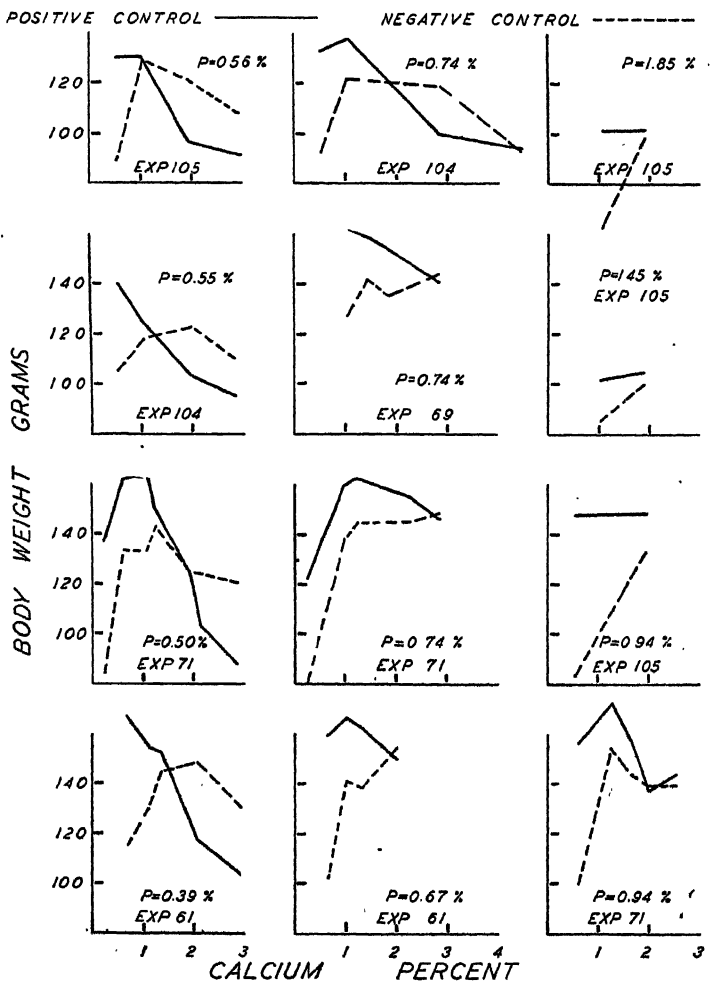


FIG. 1. Influence of Calcium on Growth, with and without Vitamin D.

between negative and positive controls was most marked in diets with lower levels of mineral. At high ratios the tendency was for vitamin D to depress the body weight.

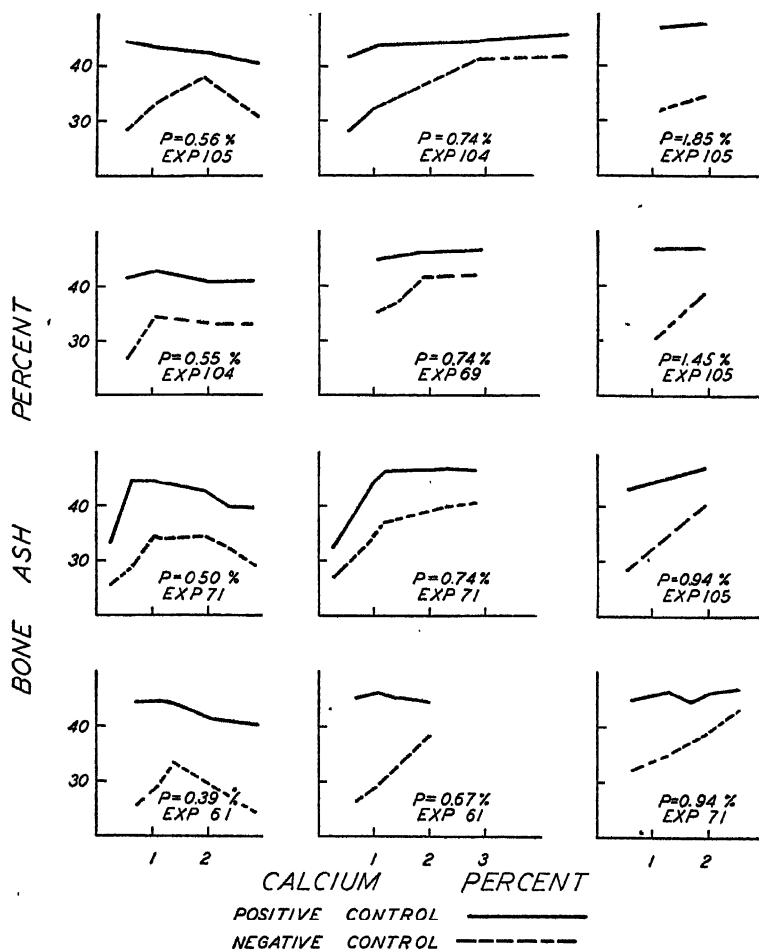


FIG. 2. Influence of Calcium on Bone Ash, with and without Vitamin D.

The effect of increasing the level of calcium and maintaining a constant level of phosphorus and thus increasing the Ca:P ratio is illustrated in Figs. 1 and 2.

The *per cent* bone ash of negative controls rose initially as the level of calcium was increased. When the phosphorus level was low, *i.e.*, 0.5%, still greater increments of dietary calcium resulted in a decrease in *per cent* bone ash. The *per cent* bone ash of the positive controls remained unchanged with increasing calcium content, with two exceptions. When the dietary calcium was increased from very low levels, the bone ash improved, and when the dietary phosphorus was low and the Ca:P ratio high, the bone ash tended to decrease.

The body weight of the negative controls tended to rise with increasing calcium level but at the higher Ca:P ratios the body weight tended to fall. The body weight of chicks in positive control groups was improved by increasing the dietary calcium from initially low levels. The depressing effect of the higher Ca:P ratios, however, was more marked than it was with the negative controls, so that in many cases the birds which received vitamin D weighed less than their mates which did not. This depressing effect of vitamin D on the weight when the Ca:P ratio was high was quite marked.

The effect of increasing the phosphorus level and maintaining a constant calcium level is illustrated in Table III.

There was little difference between negative or positive control groups except when the level of phosphorus brought the Ca:P ratio below 1 or beyond 5: this caused a fall in the *per cent* bone ash in both negative and positive control groups and a fall in the body weight of the positive control groups.

## DISCUSSION

The foregoing data confirm the work of Bethke *et al.* and Hart *et al.* (*loc. cit.*). It is quite clear that the action of vitamin D cannot be considered without reference to the calcium and phosphorus content of the diet. The varied effect on the chick, of both level and ratio of calcium and phosphorus may provide an aid in establishing the exact role and site of action of vitamin D. In the chick, the vitamin exerts its most spectacular effect on both bone ash and body weight with diets containing less than 1% of calcium, especially when the Ca:P ratio is less than 1. With high calcium-low phosphorus diets only the bone ash is improved while the body weight is depressed. On diets between the two extremes, vitamin D has no great influence on the body weight but has an appreciable effect on the bone ash.

TABLE III

*Effect of Increasing the Dietary Level of Phosphorus at a Constant Level of Calcium*

Exp. No.	Ca	P	Ca/P	Bone ash		Body weight		Necessary difference at $P=0.01$	
				Negative control	Positive control	Negative control	Positive control	Bone ash	Body weight
	<i>per cent</i>	<i>per cent</i>		<i>per cent</i>	<i>per cent</i>	<i>g.</i>	<i>g.</i>	<i>per cent</i>	<i>g.</i>
71	0.63	0.52	1.2	29.01	44.68	132.9	161.0	2.24	18.9
	0.62	0.92	0.7	30.96	45.04	100.6	156.1		
71	1.07	0.51	2.1	34.57	44.60	133.0	159.9	2.19	18.5
	0.99	0.81	1.2	34.46	44.75	136.0	160.6		
71	1.23	0.52	2.4	34.11	44.13	144.4	148.1	2.30	19.4
	1.21	0.69	1.8	37.12	46.46	143.0	161.7		
	1.29	0.96	1.3	35.15	46.66	154.0	171.7		
71	1.95	0.48	4.1	34.66	43.00	124.7	123.0	2.09	17.6
	2.03	0.95	2.1	39.40	46.18	139.4	137.0		
71	2.39	0.49	4.9	32.40	39.97	123.9	102.7	2.09	17.6
	2.28	0.72	3.2	39.90	46.98	144.5	155.0		
71	2.88	0.50	5.8	29.02	39.61	120.2	88.0	2.24	18.9
	2.84	0.77	3.7	40.69	46.85	148.3	145.7		
	2.57	1.0	2.6	43.44	47.02	138.8	144.0		
105	0.55	0.59	0.93	28.65	44.51	89.4	130.3	1.97	16.4
	0.55	0.94	0.59	28.52	43.08	83.3	147.9		
105	1.05	0.58	1.83	33.71	43.86	128.5	130.1	2.27	18.9
	1.01	0.79	1.28	31.70	45.79	126.2	147.4		
	1.04	1.5	0.69	30.55	46.83	85.5	121.8		
	1.05	1.78	0.59	31.95	46.88	62.6	101.5		
105	1.95	0.55	3.55	38.05	42.64	121.6	96.7	1.97	16.4
	1.96	0.94	2.09	40.20	47.16	132.9	148.4		
	1.91	1.4	1.36	38.74	46.92	120.0	124.2		
	1.90	1.92	0.99	34.62	47.57	98.6	101.8		



Several of the diets used above appear to have promise for the chick assay of vitamin D, but the reaction of the chick to a single optimum level of the vitamin is not necessarily a reflection of its reaction to a number of increasing suboptimal levels; nor will one trial with several levels of vitamin D suffice to establish the fact that one diet is superior to another in providing a steeper slope or smaller error in the dose response curve (*cf.* Jones, 1946).

No attempt has been made to discuss our observations in the light of prevailing theories on the fundamental action of vitamin D, nor has any histological differentiation been made of the types of "low bone ash" observed. This is being left for a subsequent paper.

#### ACKNOWLEDGMENT

The technical assistance of Mr. P. M. Richard is gratefully acknowledged.

#### SUMMARY

Experiments designed to study the effect of vitamin D on chicks which were fed diets differing in calcium and phosphorus content, indicated that:

1. The level of calcium and phosphorus in the diet affects the bone ash and body weight of chicks and the amount of vitamin D required to raise bone ash and body weight.
2. Increasing the Ca:P ratio at a given level of phosphorus raises the *per cent* bone ash and body weight which, however, fall with further additions of calcium.
3. Increasing the ratio at a given level of calcium does not affect the bone ash materially except at high levels of calcium when the bone ash falls with increasing ratio. The body weight rises with increasing ratio at lower levels of calcium and tends to fall at higher levels of calcium.
4. Vitamin D raises the bone ash of chicks on all diets.
5. Vitamin D exerts its greatest effect on the body weight on high phosphorus-low calcium diets and has a depressing effect on the body weight of chicks on high calcium-low phosphorus diets.

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# Interaction of Calcium, Phosphorus and Vitamin D.

## II. The Rachitogenic Index<sup>1</sup>

B. B. Migicovsky and A. R. G. Emslie

*From the Vitamin and Physiological Research Lab.,  
Experimental Farm, Ottawa, Canada*

Received February 3, 1947

### INTRODUCTION

The variations in response to graded doses of vitamin D have been noted by Jones (1945) and Bliss (1946). Although a study of the response to optimal doses of vitamin D by Migicovsky and Emslie (1947) revealed that there was not a great deal of difference between the various diets, the difference in the negative controls indicated that a number of the diets would elicit a different response to a series of suboptimal levels of vitamin D.

The following experiments were planned to demonstrate the effect of the calcium and phosphorus content of the diet on the relative position and slope of the dose-response curves for three common criteria of rickets in the chick.

### EXPERIMENTAL

The basal diet used in this investigation was the A.O.A.C. (1940) ration without the  $\text{Ca}_3(\text{PO}_4)_2$ . The calcium and phosphorus content was adjusted by adding appropriate amounts of  $\text{CaCO}_3$  and  $\text{KH}_2\text{PO}_4$ .

The vitamin D was supplied as Canadian Standard Reference Oil, appropriate amounts of which were diluted with corn oil and mixed into the diet at a level of 1%.

The diets employed are classified as A.O.C.A., high-calcium and low-calcium. The calcium and phosphorus content of the individual diets are shown in Table I. It should be noted that the calcium content of the low-calcium diet, while lower than is usual in this laboratory for the A.O.A.C. diet, is not so low as to cause calcium starvation when sufficient vitamin D is administered.

The experiments were designed so that a comparison could be made between the A.O.A.C. diet and one or both of the other two. The A.O.A.C. diet was fed to quadrup-

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<sup>1</sup> Technical Contribution No. 131, Division of Chemistry, Science Service, Department of Agriculture, Ottawa, Canada.

TABLE I

*Calcium and Phosphorus Content of Diets*

Exp. No.	A.O.A.C.		High-calcium		Low-calcium	
	Ca	P	Ca	P	Ca	P
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
107	1.05	0.68	2.75	0.47	0.69	0.65
108	0.83	0.77	2.30	0.51		
109	0.87	0.75	2.74	0.50		
111	1.01	0.79	2.97	0.60	0.53	0.79

plicate groups in each of four experiments whereas the high-calcium and low-calcium diets were fed to triplicate or duplicate groups in 4 and 2 experiments, respectively.

One-day old white leghorn chicks were used in groups of 15 to 20. The chicks were on experiment for 3 weeks, at the end of which time they were chloroformed, X-rayed, and the left tibiae and both middle toes removed. The tibiae were cleaned, extracted and ashed in groups as described in the A.O.A.C. (1945) chick assay for vitamin D. Toes were dried and ashed.

All diets were analyzed for calcium and phosphorus, using perchloric acid digestion followed, in the case of calcium, by permanganate titration and, in the case of phosphorus, by colorimetric estimation.

## RESULTS

The response of chicks to graded doses of vitamin D was measured by the ash percentage of tibiae and dried toes and by the log (tarso-metatarsal distance  $\times 13$ ). These values are given in Table II.

The dose-response curves for A.O.A.C., high- and low-calcium diets are shown in Fig. 1. Application of the chi square test indicates they are parallel. Since the potency of the reference standard cannot change, the horizontal distance ( $M$ ) between the A.O.A.C. and either of the other two dose-response curves, which was calculated by the method of Irwin (1937), must represent the difference in relative rachitogenic activity of the diets. To assign a value to this activity we formulate the

rachitogenic index which is equivalent to  $\frac{1}{\text{antilog } M}$ ,  $M$  being the term expressing the relationship between two dose-response curves. The values for the rachitogenic indices of the diets under consideration are given in Table III.

TABLE II

*Response of Chicks to Graded Doses of Vitamin D on A.O.A.C.,  
High-Calcium, and Low-Calcium Diets*

Exp. No.	Dose	Tibia ash			Toe ash			Log. T.M.T.		
		A.O.A.C.	High-Ca	Low-Ca	A.O.A.C.	High-Ca	Low-Ca	A.O.A.C.	High-Ca	Low-Ca
	<i>units</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>			
107	0	31.69	33.67	27.49	10.01	9.66	8.33	1.490	1.443	1.526
	9	36.64	32.02		11.76	8.71		1.286	1.573	
	15	41.20	35.49	32.02	13.01	9.26	9.58	1.066	1.461	1.417
	25	41.57	37.86	34.33	13.24	11.06	10.83	0.954	1.271	1.341
	41.7			36.18			11.27			1.244
108	0	31.35	31.58		10.46	9.22		1.447	1.533	
	9	36.87	33.33		11.13	9.82		1.322	1.546	
	15	37.74	35.36		12.34	10.26		1.195	1.481	
	25	40.68	39.14		13.04	12.01		0.991	1.248	
	100		39.79			12.48			1.184	
109	0	32.99	36.16		9.62	11.43		1.322	1.335	
	9	36.30	36.01		11.52	11.61		1.195	1.293	
	15	39.58	38.09		12.58	12.14		1.039	1.149	
	25	40.72	38.52		13.11	11.96		0.786	1.058	
	100		40.69			13.68			0.945	
111	0	33.43	34.86	29.44	10.44	10.04	9.24	1.322	1.408	1.398
	9	38.41	36.30		12.10	10.79		1.024	1.346	
	15	39.97	36.83	30.39	11.98	11.12	9.87	0.994	1.286	1.382
	25		40.75	32.19		12.30	10.27		1.070	1.324
	41.7			35.53			11.99			1.230
	100		43.14	39.02		12.72	12.12		0.894	1.007

The results show that the rachitogenic effect of the low-calcium diet is greater than that of the high-calcium diet which is, in turn, greater than the rachitogenic effect of the A.O.A.C. diet, the index of which is taken as 1.

Since the rectilinear portions of the log dose-response curves are parallel we must conclude that the antirachitic action of vitamin D is independent of diet. The diets differ in the degree of "rickets" in the negative control and in the amount of vitamin D required to deflect the dose-response curve. These two factors are mainly responsible for

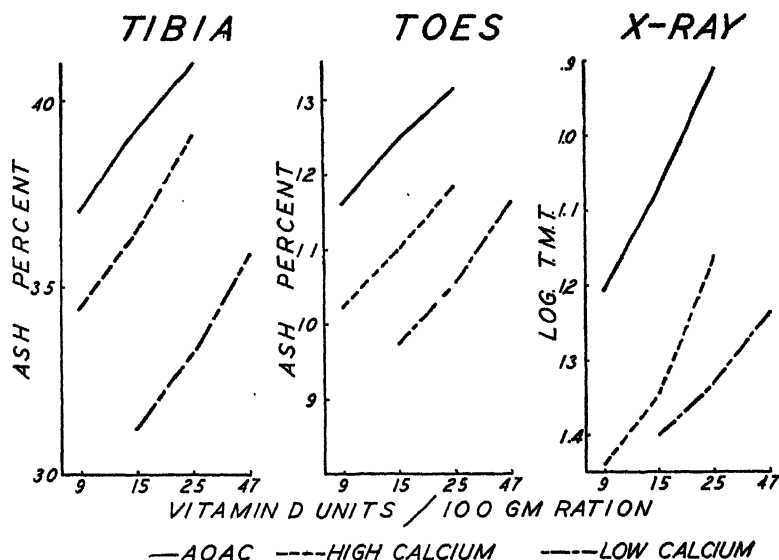


FIG. 1. Log. Dose-Response Curves of A.O.A.C., High-Calcium and Low-Calcium Diets for 3 Criteria of Response.

the rachitogenic indices given in Table III. It appears, therefore, that the dose-response curve is made up to two phases. In one, little or no calcified bone is laid down, and the extent of this phase is dependent on the relative quantities of calcium and phosphorus furnished to the system. The second phase is concerned with bone deposition, which

TABLE III  
*Rachitogenic Index (R.I.) of High- and Low-Calcium Diets  
Relative to A.O.A.C. Diet*

Criterion	High-calcium diet		Low-calcium diet	
	R.I. and error <sup>a</sup>	$\chi^2$ test for parallelism <sup>b</sup>	R.I. and error <sup>a</sup>	$\chi^2$ test for parallelism <sup>b</sup>
Tibiae	1.82 (1.34-2.47)	0.0380	6.38 (3.98-10.31)	0.0061
Toe	2.51 (1.57-4.00)	0.1250	5.00 (3.92- 6.41)	0.1112
T.M.T.	2.44 (1.44-4.13)	0.0200	4.81 (2.94- 8.13)	3.1600

<sup>a</sup> Limits of error are calculated from the standard deviation for months at  $P = 0.05$ .

<sup>b</sup> Critical value for significance at  $P = 0.05$  is 3.841.

proceeds when the initial vitamin D requirement is met. This lends credence to the theory that vitamin D acts in the preparation of mineral for bone deposition.

It is interesting to note that, when the log rachitogenic index of the diets are plotted against the response for a given dose of vitamin D, the resultant curve is a straight line and is parallel to similar curves for different doses of the vitamin. This suggests an alternate method of assay for vitamin D which might be more precise. Studies along this line are in progress.

The data in Table III also indicate that the rachitogenic index of the high-calcium diet is less when calculated on the basis of *per cent* ash of tibia than when either toe ash or T.M.T. is the criterion of rickets. On the low-calcium diet there is no difference between the three criteria. The only explanation we have to offer is that the high calcium diet brings about a degree of mineralization in the shaft of the tibia independent of the vitamin D. This effect would not be noticeable in toe ash or T.M.T. distance.

#### ACKNOWLEDGMENT

The technical assistance of Mr. P. M. Richard and Mr. L. J. Carter is gratefully acknowledged.

#### SUMMARY

Chicks were fed A.O.A.C., high-calcium and low-calcium diets, containing graded amounts of vitamin D. No difference was found in the slopes of the dose-response curves. The relative rachitogenic effect of the three diets for three criteria of response is described by means of the "rachitogenic index."

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# Enzymatic Hydrolysis of Desoxyribonucleic Acid

Charles A. Zittle

*From the Biochemical Research Foundation, Newark, Delaware*

Received January 27, 1947

## INTRODUCTION

Desoxyribonucleic acid can be readily hydrolyzed to nucleosides and phosphoric acid by the successive action of a specific nuclease, recently purified by McCarty (1), and phosphoesterase from calf intestinal mucosa (2). The nuclease appears to form tetranucleotides (3) from the nucleic acid and the phosphoesterase hydrolyzes these stepwise to mononucleotides and nucleosides (4). The release of secondary phosphoric acid groups during the first two stages of the hydrolysis, which has been measured manometrically and titrimetrically and is reported herein, conforms to such a formulation. The action of the phosphoesterase has been followed also by the increased solubility of the hydrolyzed substrate in the uranium reagent (5, 6) and by the release of phosphoric acid. Experiments are described which indicate that the phosphoesterase can partially hydrolyze desoxyribonucleic acid without preliminary hydrolysis by the nuclease, although at a very slow rate.

## EXPERIMENTAL

### *Preparation of Reagents*

The phosphoesterase was prepared from calf intestines and assayed by the methods described previously (7).

The nuclease was extracted from beef pancreas and precipitated by 0.4 saturation with ammonium sulfate by McCarty's procedure (1). The precipitate was dried in a vacuum desiccator and its activity determined at pH 7.5 from its ability to hydrolyze the substrate nucleic acid to compounds soluble in 0.25 *N* HCl, tested by mixing equal volumes of the treated 0.6% nucleic acid and 0.5 *N* HCl and observing the amount of precipitate or the increase in soluble P. The nuclease was used without further purification except for the manometric experiments when it was dialyzed against 0.0005 *N* H<sub>2</sub>SO<sub>4</sub> to remove the ammonium sulfate.

Desoxyribonucleic acid was prepared from calf thymus by the method of Hammarsten (8), although the temperature was not maintained at 0°C. for all the operations. The preparation was completely precipitated by HCl.

### *Action of Nuclease on Desoxyribonucleic Acid*

One part of the nuclease preparation, a considerable portion of which is ammonium sulfate, made 20 parts of the nucleic acid completely soluble in 0.25 *N* HCl in about an hour at 20–25°C. at pH 7.5. The increase in HCl solubility was negligible when sodium citrate was added. According to McCarty (1) the effect of citrate is due to the binding of magnesium which activates the nuclease. In the course of the hydrolysis the solubility of the nucleic acid-P in the uranium reagent increased to about 20%.

A release of acid groups by the action of the nuclease on the nucleic acid (3.0–9.0 mg.) was demonstrated manometrically with Warburg equipment at pH 7.62 using the methods described (7). A stable nuclease solution (0.75% initial weight), 0.5 cc. of which was used in each experiment, was prepared as follows: the dialyzed nuclease was neutralized with NaHCO<sub>3</sub>, neopeptone was added (1.3%) to prevent destruction of the nuclease by proteolytic enzymes (1), and magnesium chloride was added (0.017 *M*) for activation. The CO<sub>2</sub> released by the action of the nuclease was corrected for dilution effects and retention (7). The active enzyme released 105 mm<sup>3</sup>. of CO<sub>2</sub>/0.72 mg. of nucleic acid-P; there was no release of CO<sub>2</sub> with 0.03 *M* sodium citrate present. The amount of acid released is 0.81 of the amount calculated for one acid group for each four P atoms of the nucleic acid.

Manometric measurement of the rate of release of acid can serve as another (1, 9) assay procedure for this enzyme. With 12 mg. of nucleic acid the rate of acid release for a 10-minute period was proportional to the amount of nuclease preparation used (0.4–1.1 mg.) In a recent report such an assay procedure has been utilized for this enzyme (10).

### *Action of Phosphoesterase on Desoxyribonucleic Acid*

With 2.0 mg. of phosphoesterase and 6.0 mg. of substrate the initial rates of hydrolysis of the untreated and nuclease-treated nucleic acids, measured manometrically at pH 8.14 (7), were respectively 16 and 87 mm.<sup>3</sup>/10 min. The possibility that the phosphoesterase preparation contained nuclease which first acted on the substrate was eliminated by repeating the experiment at pH 7.62 with thoroughly dialyzed phosphoesterase with and without magnesium and with sodium citrate. The magnesium did not increase the activity as it would if nuclease were participating, nor did the presence of citrate (0.01 and 0.03 *M*) have a significant effect on the activity.

Differences in the effect of phosphoesterase on untreated and nuclease-treated nucleic acid are shown strikingly in Fig. 1. Four times as much enzyme was used for the former as the latter. The course of

hydrolysis of the former was not altered perceptibly by the presence of 0.01 *M* sodium citrate. The solubility in the uranium reagent of the untreated nucleic acid after action of phosphoesterase was not complete, attaining only 70% (average of 4 experiments), even after prolonged action of the enzyme. Successive treatment of this resistant fraction, however, with nuclease and phosphoesterase made it readily soluble.

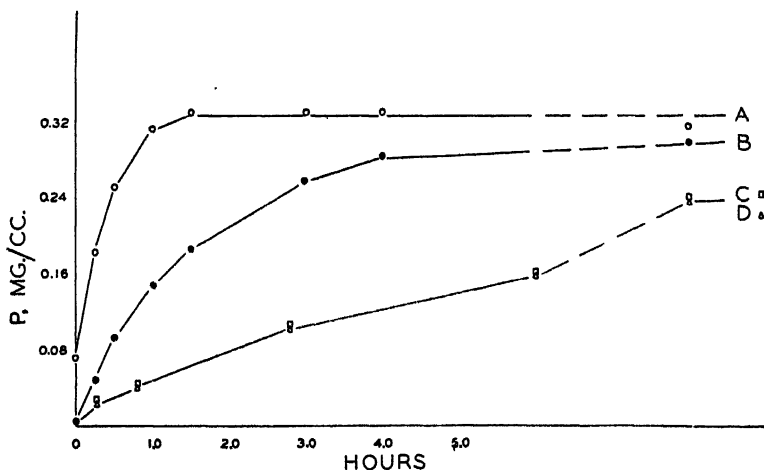


FIG. 1. Action of Phosphoesterase on Untreated and Nuclease-treated Desoxyribonucleic Acid. 30.0 cc. of a 0.6% solution of nucleic acid were buffered with 3.0 cc. of 0.5 *M*  $\text{NaHCO}_3$  and enzyme added (15.0 mg. to the treated and 60.0 mg. to the untreated nucleic acid) in a volume of 10.0 cc. The experiments were performed at room temperature. Immediately after adding enzyme, and subsequently at intervals, a sample was taken and mixed with an equal volume of uranium reagent (6). The precipitate was removed by centrifuging and total P and inorganic phosphate-P determined on the supernatant fluid. Nuclease-treated nucleic acid (total P = 0.320 mg./cc.): Curve A, total soluble P; Curve B, inorganic phosphate-P. Untreated nucleic acid (total P = 0.288 mg./cc.): Curve C, total soluble P; Curve D, inorganic phosphate-P.

Acid groups released by action of phosphoesterase on nuclease-treated desoxyribonucleic acid were estimated by the manometric procedures which have been described (7). Typical experiments with the nuclease-treated nucleic acid gave the following results. In an experiment at pH 7.62 with silver nitrate present (0.00017 *M*) to inhibit the adenosine deaminase (11) the phosphoesterase acting on 0.111 mg.

of uranium reagent-insoluble P liberated 63 mm.<sup>3</sup> of CO<sub>2</sub>; this is equivalent to 3.1 secondary phosphoric acid groups per tetranucleotide-residue (four P atoms). A similar experiment without silver nitrate but corrected for deamination (7) gave the value 2.7. Experiments performed at pH 8.14 with 0.144–0.288 mg. of uranium reagent-insoluble P and the results corrected for carbonate and dilution (7) gave an average value of 2.9 groups per tetranucleotide-residue. Since the data were in agreement within the limits of accuracy of the methods, it was concluded that three acid groups are liberated by the hydrolysis of a tetranucleotide-residue.

The titrimetric experiments were performed as before (7). Based on the differences in the inflection points of the titration curves (Fig. 2) for the unhydrolyzed and hydrolyzed material  $3.2 \pm 0.2$  secondary phosphoric acid groups were liberated per tetranucleotide-residue (3 experiments); based on the NaOH required to restore the hydrolyzate to its initial pH value the number of acid groups liberated was 10% greater.

## DISCUSSION

The demonstration by manometry of the liberation of acid (0.81 equivalent/tetranucleotide-residue) by the action of nuclease on desoxyribonucleic acid is in agreement with Fischer *et al.* (3), who found by titrimetry that one secondary phosphoric acid group is liberated for each four P atoms present. Carter and Greenstein (12) recently reported a value of similar magnitude; purified nuclease released  $5.4 \times 10^{-7}$  equivalents of acid, measured by titration, from 1.0 mg. (0.086 mg. P) of nucleic acid, equivalent to 0.78 moles/tetranucleotide (four P). This is contrary to earlier studies from the same laboratory—with tissue extracts containing this enzyme no change in pH was observed when they acted on desoxyribonucleic acid (13).

According to Fischer *et al.* (3) tetranucleotides are formed by the action of the specific nuclease on desoxyribonucleic acid. In the present study, insolubility in the uranium reagent (5, 6) is taken as a measure of tetranucleotides since mononucleotides are soluble in this reagent (5). This may be subject to some error since the behavior of di- or trinucleotides in this reagent is now known; furthermore, the nature of the fraction (20%) that, in the present study, became soluble in the uranium reagent by the action of the nuclease remains to be clarified.

The data, however, on either the basis used or on the basis of total nucleic acid-P show that, in round numbers, three secondary phosphoric acid groups are liberated when the product obtained by the action of the nuclease is completely hydrolyzed by the phosphoesterase.

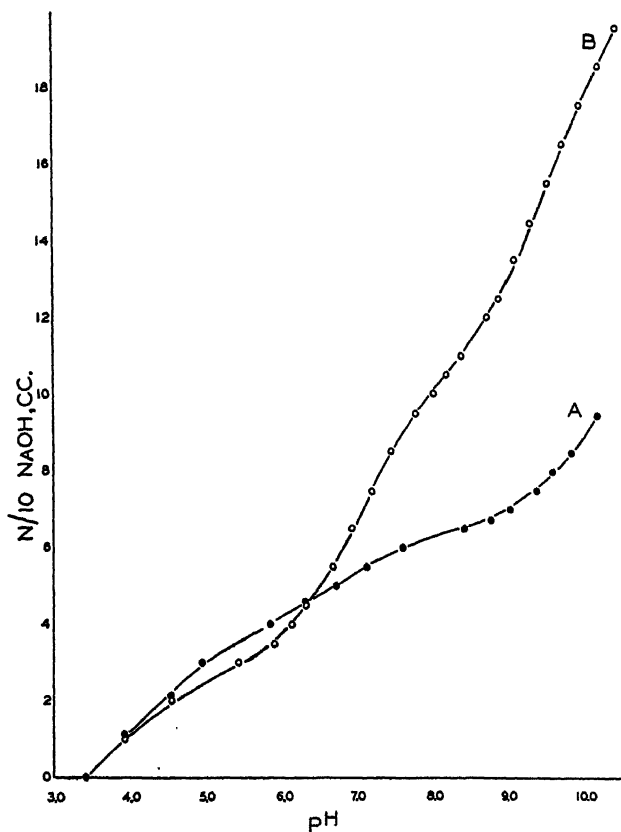


FIG. 2. Titration Curves of Nuclease-treated Desoxyribonucleic Acid before and after Phosphoesterase Hydrolysis. The nucleic acid sample contained 15.5 mg. of P insoluble in the uranium reagent, equivalent to 0.000125 moles of tetranucleotide-residue (four P). Curve A, unhydrolyzed; Curve B, hydrolyzed nucleic acid.

The data indicate that all of the mononucleotides are bound to each other through secondary phosphoric acid groups, which is in agreement with the formula for desoxyribonucleic acid proposed by Levene in 1921 (14) from other evidence.

The experimental data indicate that phosphoesterase can hydrolyze a part of the nucleic acid molecule even though bonds hydrolyzed by the specific nuclease are still intact, but at a much slower rate, although the bond acted on by the nuclease appears not to be broken by the phosphoesterase. This would be observed, of course, if the phosphoesterase preparation contained nuclease; this is unlikely since McCarty (1) found that nuclease is destroyed by proteolytic enzymes and they are used in preparing the phosphoesterase. Convincing evidence that contamination of the phosphoesterase with nuclease is not responsible for the hydrolysis is the fact that hydrolysis occurs in the presence of citrate. Phosphoesterase has been reported (15, 16, 17) not to act directly on deoxyribonucleic acid, but it appears from the above considerations that the difference in the results with the untreated and nuclease-treated nucleic acids is quantitative and not qualitative. The divergent results may be due to a difference in the degree of non-specific, non-enzymatic depolymerization (18) and it is understood that the above conclusion implies only that the bonds of the nucleic acid hydrolyzed by the nuclease may be intact and yet the phosphoesterase can bring about considerable hydrolysis.

Other evidence for the above conclusion is the fact that phosphoesterase will slowly hydrolyze deoxyribonucleic acid prepared by the method of Levene (19) and yet the nuclease has little effect on it. The resistance of this nucleic acid to the nuclease appears to concern the adenine radical (20).

#### SUMMARY

Four secondary phosphoric acid groups were found to be liberated per tetranucleotide-residue in the hydrolysis of deoxyribonucleic acid by the successive action of specific nuclease and phosphoesterase, measured by manometry and titrimetry. Phosphoesterase alone hydrolyzed deoxyribonucleic acid slowly to the extent of 70%, but after preliminary treatment of the nucleic acid with the specific nuclease the action of the phosphoesterase was rapid and hydrolysis was complete.

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# Crystalline, High-Molecular-Weight Compound from Commercial Pepsin

Jaques Bourdillon

*From the Division of Laboratories and Research,  
New York State Department of Health, Albany*

Received February 10, 1947

## INTRODUCTION

Commercial pepsin (Parke, Davis, and Company, 1:10,000) contains a crystalline substance of rather unusual character, which appears to be a high-molecular-weight, pepsin-resistant polypeptide, and for which the term *peptophan* is here proposed. The present paper offers a study of the main properties of this substance.

## ISOLATION

Two hundred g. of commercial pepsin (Parke, Davis, 1:10,000) were dissolved in 400 ml. of water. The pH was adjusted to 7.5 with 1 *N* NaOH and the solution boiled for a few minutes, using a wide beaker and stirring vigorously to avoid boiling over. The pH was then adjusted to 4.0 with 1 *N* HCl and the solution boiled again for a few minutes, cooled, and filtered through coarse paper. To the clear filtrate, 0.8 volume of saturated ammonium sulfate solution was added. After two days at room temperature in a tall container to allow the precipitate to settle (the precipitate formed but slowly), the supernatant was siphoned off and the precipitate sedimented by centrifugation. It was washed in 100 ml. of 45% saturated ammonium sulfate solution which was 0.1 *M* in acetate buffer, pH 4.6, and centrifuged. This operation was repeated twice. Finally, enough water was added to the precipitate to yield a fairly fluid suspension, which was transferred to a section of cellophane tubing and dialyzed against distilled water at 5°C.

## CRYSTALLIZATION

The brown solution thus obtained usually contained a precipitate which, on microscopic examination, was found to consist of material ranging from rather characteristic irregular masses (Fig. 1a) to tightly packed clusters of plates or sharp needles (Fig. 1b). As soon as the solution was heated above 60°C., these masses dissolved, leaving a clear solution with little or no insoluble material. Upon cooling, the same masses would appear after a few hours. This material is referred to as crude *peptophan*.

To obtain a larger yield of crystals, the procedure was as follows: To 10 ml. of a 5% crude peptophan solution was added 1 ml. of *M* acetate buffer, pH 4.6, and 0.5 ml. of saturated ammonium sulfate solution. After a few hours at room temperature, a precipitate of very fine, irregular, flat needles appeared. The crystals thus obtained are so thin and small that they lend themselves but poorly to accurate photographing. (Fig. 1c.) Addition of saturated ammonium sulfate in small portions was continued over a period of 2-3 days until a saturation of 20% was reached. Further addition of ammonium sulfate caused the precipitation of amorphous material. The crystals were separated by centrifugation, washed in 20% saturated ammonium sulfate, pH 4.6, suspended in a little water, dissolved by heating, and dialyzed against distilled water

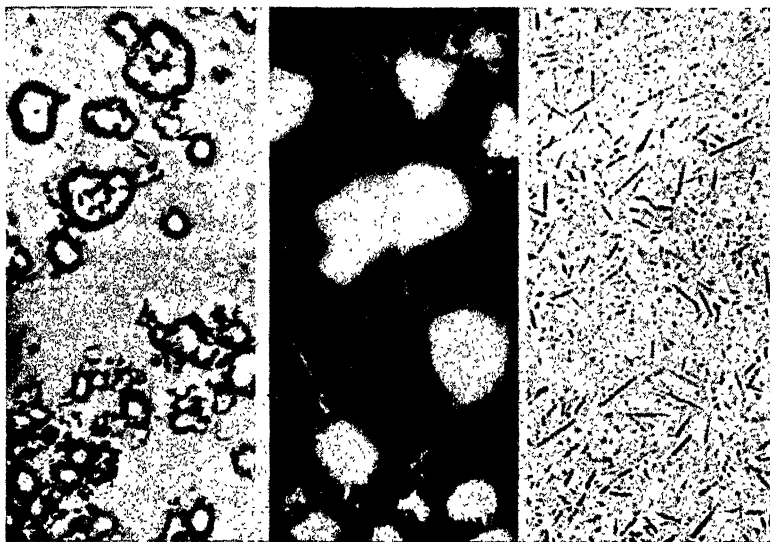


Fig. 1. Peptophan. (a) Semicrystalline precipitate in cold water; (b) Clusters and single needles in cold water; (c) Needles in ammonium sulfate solution. ( $\times 400$ ).

at 5°C. After a few days, the solution again showed a heavy precipitate of single needles and clusters. The yield could be increased by addition of a little ammonium sulfate.

The yield of crude peptophan from 200 g. of pepsin was about 3 g., that of once-crystallized peptophan, 0.5-1 g.

#### GENERAL PROPERTIES

Crude peptophan solutions in water are yellowish brown; they may be turbid because of partial precipitation of the material but clarify as soon as heated. Once-crystallized peptophan has a greenish tinge,

dissolves less readily, and requires a little alkali for complete solution. Crystallized peptophan is stable in 0.1 *N* HCl and in 0.1 *N* NaOH for at least 24 hours at room temperature and recrystallizes upon dialysis. Boiling at neutral pH causes no alteration. It may be dried at room temperature to glassy brown flakes that are appreciably hygroscopic. Drying at 105°C. in air impairs somewhat the ability to crystallize.

Peptophan is precipitated by about 50% alcohol, acetone, or dioxane, by phosphotungstic and by picrolonic acid. It is incompletely precipitated by tungstic, picric, or cold trichloroacetic acids, not precipitated by glacial acetic nor by hot trichloroacetic acid. It is completely precipitated by 50% saturation with ammonium sulfate at slightly acid pH but the precipitation is rather slow. In all cases, the solubility increases rapidly with temperature.

### CHEMICAL PROPERTIES

Some essential chemical information is given in Table I. The nitrogen and carbon percentages, the absence of phosphorus and carbohydrate, and the positive biuret suggest a polypeptide. The positive reaction with Folin's reagent is strong and occurs without the addition of copper, which, in the absence of tryptophan, would indicate that the reaction is due to tyrosine itself (1). The nitroprusside test is positive in the crude material alone, and only if the latter is first heated with KCN. Since addition of cyanide is necessary for a positive test with cystine and with some proteins or protein degradation products that do not

TABLE I  
*Chemical Properties of Peptophan*

	Crude material	Once-crystallized
Nitrogen (Kjeldahl) ( <i>per cent</i> )	16.6	16.1
$\alpha$ -Amino nitrogen (Van Slyke) ( <i>per cent</i> )	0.5	
Carbon (Van Slyke and Folch) ( <i>per cent</i> )	48.9	49.9
Phosphorus (phosphomolybdate)	—	
Tyrosine-tryptophan (Folin)	+	+
Tryptophan (Hopkins-Cole)	—	—
Carbohydrate (Molisch) ( <i>per cent</i> )	3-5	—
SH (nitroprusside)	+	—
Biuret	+	+
HNO <sub>3</sub>	no color	

give it on simple boiling, it would appear that crude peptophan contains some sulfur in the oxidized state while crystalline peptophan contains none. There is not enough sulfur, however, to cause blackening upon heating with NaOH and lead acetate. Crystallized peptophan was found to contain a trace of iron (1 part in 5000 by the dipyrldyl method). The presence of other metals was not investigated. Addition of heavy metal salts in high concentration appeared to degrade the substance. The scarcity of commercial pepsin has as yet prevented a more extensive chemical study from being undertaken.

### PHYSICOCHEMICAL PROPERTIES

Peptophan solution could be dialyzed in cellophane for long periods without loss, and the fact that the bag failed to swell appreciably, even with concentrated solutions, suggests that the substance is usually in a high state of aggregation (average molecular weight of the order of at least  $10^5$ ). It was, unfortunately, impossible to obtain stable osmotic pressures by the author's method (2) and the time-pressure curves observed were so erratic as to suggest that the substance was physically unstable, even though, in 0.1 *M* phosphate buffer, pH 7.3, the solutions remained perfectly clear. Any more definite statement on the molecular weight must, therefore, await a study of the sedimentation rate.

A 2.25% solution of crude peptophan was found to have a relative viscosity of 1.58 at 25°C. The refractive index of the same material was such that each scale division of the Zeiss dipping refractometer corresponded to a concentration of 0.208%, which yields a factor *a* of about 0.00185, the same as for horse serum proteins (3).

The acid- and base-binding power of the substance is shown in Fig. 2. The curve was constructed on the assumption that the number of acidic or basic valences was represented by the difference between the number of H or OH ions added and those accounting for the pH of the solution. HCl and NaOH were used in salt-free solutions. Measurements were made with a glass electrode, and the values obtained on the alkaline side were corrected (according to the chart provided by the manufacturer) for the sodium error of the electrode. The curves obtained with crude and with crystallized preparations do not differ materially. The isoelectric point is between pH 5 and 6, and both curves show a slight inflection at pH 3.

## BIOLOGIC PROPERTIES

The evidence thus far is mostly negative. Peptophan, as its very presence in commercial pepsin suggests, appears to be completely resistant to peptic hydrolysis. If the crystalline material is allowed to stand for 24 hours with 1:100 its weight of commercial pepsin at pH 1.5, it will recrystallize as soon as neutralized and dialyzed, with no apparent loss.

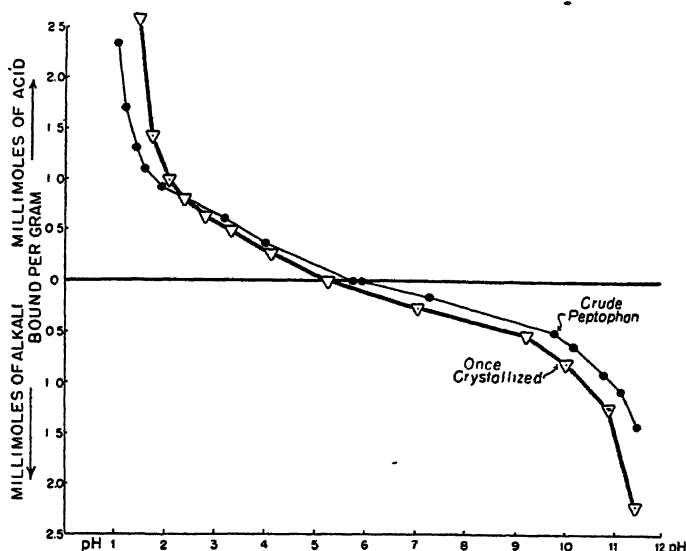


FIG. 2. Acid and Base Binding of Peptophan upon Addition of HCl or NaOH in Salt-Free Solution.

Neither does commercial trypsin appear to attack peptophan to an appreciable extent. When crystalline peptophan was allowed to stand for 24 hours at room temperature with 1:100 its weight of trypsin at pH 8.5, it failed to recrystallize upon simple dialysis, and did so only when the ammonium sulfate method was used. Crystallization was imperfect, the material consisting of clusters and spheroids with but few loose needles. This may have been due as much to the presence of trypsin in the solution as to some slight hydrolysis of the material. Table II shows that even crude peptophan failed to be extensively attacked by trypsin, the free  $\alpha$ -amino nitrogen rising from 3 to only 10% of the total nitrogen of the substance.

TABLE II  
*Tryptic Digestion of Crude Peptophan*

Peptophan was dissolved in distilled water. The enzyme was added in the form of a filtered solution of 1:110 Pfanstiehl trypsin; pH adjusted with NaOH.

Trypsin	pH	Pepto- phan N	$\alpha$ -amino N		Time
			mg./ml.	per cent of peptophan N	
mg./ml.		mg./ml.			
	(no NaOH)	0.86	0.023	2.7	No incubation
	8.5	0.86	0.026	3.0	16 hours at 35°C.
0.025	(no NaOH)		0.001 (approx.)		No incubation
0.025	8.3		0.020		16 hours at 35°C.
0.025	8.3	0.86	0.107	10.1*	16 hours at 35°C.

\* After subtraction of 0.020 mg./ml.  $\alpha$ -amino N due to the autolysis of trypsin.

Peptophan seems to bear no relation to the enzyme pepsin itself. Commercial pepsin was allowed to stand for a week at 35°C. at pH 1.3, conditions which should cause marked hydrolysis of the enzyme (4). The fraction precipitated at 45% ammonium sulfate saturation after removal of the enzyme itself by denaturation was not found to increase under such conditions but, on the contrary, to decrease slowly. This indicates that direct acid hydrolysis of pepsin does not produce material precipitated by 45% saturation with ammonium sulfate, and that the peptophan present is slowly destroyed. Pepsin first denatured by short boiling at pH 7.5 and precipitated at pH 4.0 (as in the preparation of peptophan) appeared remarkably resistant to hydrolysis upon addition of fresh pepsin at pH 1.5. If the boiling lasted 90 minutes, marked hydrolysis would occur under the same conditions, but the nonprotein fraction thus produced, that precipitated at 45% ammonium sulfate saturation, appeared to be completely different from peptophan.

Peptophan was not found to inhibit peptic activity in the digestion of horse diphtheria antitoxic pseudoglobulin, either at pH 1.5 or at pH 3.6, under the same conditions as those of a previous investigation (Ref. 5, Table II, exp. 3).

No role could be assigned to peptophan in hydrogen transfer, using various substrates and the reduction of methylene blue *in vacuo* as an indicator.

Finally, the material was not antigenic. Three rabbits, inoculated intravenously 4 times a week with first 0.1 mg., then 1.0 mg. of crude peptophan, over a period of two months, failed to show any antibody production, either by the precipitin or by the complement-fixation test. The injections were extremely well tolerated.\* It is possible that a high-molecular-weight, pepsin- and trypsin-resistant polypeptide in the gastric mucosa plays a significant part in protecting the stomach against self-digestion.

### SUMMARY

A crystalline substance which appears to be a polypeptide has been isolated from commercial pepsin.

Qualitative evidence indicates that it contains tyrosine but no tryptophan, no sulfur, no phosphorus, and no carbohydrate.

It has an isoelectric point between pH 5 and 6 and is obtained in an extremely high state of aggregation (average molecular weight probably over  $10^6$ ).

It is pepsin-resistant and attacked but little, or not at all, by commercial trypsin. It is not antigenic. Its biologic significance has not been elucidated.

The term *peptophan* is proposed for this substance.

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\*The author is indebted to Miss Jessie L. Hendry for this part of the work.





# Urinary Excretion of Twelve Amino Acids by Normal Male and Female Subjects Measured Microbiologically\*

Max S. Dunn, Merrill N. Camien, S. Shankman  
and Harriette Block

*From the Chemical Laboratory, University of California, Los Angeles*

Received September 23, 1946

## INTRODUCTION

The two preceding studies (1, 2) on the urinary excretion of apparent free tryptophan, histidine and cystine by normal and pathological male and female subjects has been extended in the present experiments by determination of the daily urinary excretion of 12 amino acids by normal male and female subjects. It was considered desirable to determine both the free and the combined forms of these amino acids, as it was suggested previously (2) that the total tryptophan excreted may be several-fold greater than that of the free amino acid.

## EXPERIMENTAL

Twenty-four hour urine samples were preserved by the addition of toluene and storage in a refrigerator. Both untreated and hydrolyzed samples were assayed for amino acids. Samples were hydrolyzed by the following procedures:

(a) *HCl Hydrolysis*. One hundred to 200 ml. of 12 N HCl were added to the 24-hour urine, the mixture was evaporated under reduced pressure to twice the volume of the added acid and the concentrated solution was refluxed 20 hours on an oil bath. Excess HCl was removed by evaporating the final solution to dryness under reduced pressure. The residue was dissolved in boiling water and the small amount of insoluble material

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\* Paper 36. For Paper 35 see Frankl *et al.* (1). This work was aided by grants from the American Home Products Co., the John and Mary R. Markle Foundation, Merck and Co., the Nutrition Foundation, Standard Brands and the University of California. The Army K-Rations employed in this experiment were furnished by the Military Planning Division, Research and Development Branch, Office of the Quartermaster General, Army Service Forces, for use on Project No. R 49001-QM-382. The authors are indebted to S. C. Goldberg for technical assistance.

removed by filtering the suspension. The filtrate was diluted to 500 ml. and this solution was stored in the refrigerator.

(b) *Urease Hydrolysis*. One liter aliquots of 48-hour urines were treated with 2 ml. of an extract of urease tablets<sup>1</sup> and the mixture incubated for 18 hours at 40°C. under toluene. Blanks were prepared in the same manner omitting the urine aliquot.

Since the neutralized acid hydrolyzates contained relatively large amounts of sodium, ammonium and chloride ions which might affect the growth of the microorganisms, the concentration of these ions was determined by standard methods<sup>2</sup> and the solutions of standard and sample were adjusted to the same concentration of these ions. Thus the effect of the salts on the growth of the microorganisms was the same both for the sample and standard tubes.

Microbiological assay methods, essentially the same as those described by the authors cited, were employed for the determination of the following amino acids: aspartic acid (3), arginine (4), glutamic acid (5), glycine (6), histidine (7, 8), isoleucine (9), leucine (9), lysine (10), methionine (11), phenylalanine (12), threonine (13), tryptophan (9), tyrosine (4) and valine (9). A sample for the recovery of each amino acid was prepared by adding the amino acid to the untreated urine in an amount approximately equal to that present in the aliquot and subjecting the recovery solution to the same hydrolytic procedure as that used in hydrolyzing the corresponding assay sample.

## DISCUSSION

It was found (Table I, footnote 2) in the first experiments that (apparent) free tryptophan and cystine could be determined satisfactorily with *L. arabinosus* 17-5 (8) and that (apparent) free arginine, phenylalanine and tyrosine could be determined satisfactorily with *L. casei* (4). The attempted determination of the other (free) amino acids was unsatisfactory. No glutamic acid could be detected with *L. arabinosus* 17-5 (5). (Free) Leucine, isoleucine and valine could not be determined satisfactorily with *L. arabinosus* 17-5 (8) either in diluted or undiluted urines. It was considered probable that the inhibitions observed with undiluted urines were caused either by urea *per se* or by its alkaline decomposition products formed during autoclaving of the urine. If inhibitory effects were reduced by diluting the urines, the concentrations of leucine, isoleucine and valine were reduced below the limits of sensitivity of the available methods. Attempts were made to determine methionine and threonine with *L. arabinosus* 17-5 (8) but strong stimulation occurred and the recoveries (200–300 per cent) were unsatisfactory. Since it was found, subsequently, that these

<sup>1</sup> 1.5 g. of Squibb's urease tablets were triturated with 15 ml. of distilled water, the mixture was allowed to stand 30 minutes and the suspension was filtered.

<sup>2</sup> Ammonia by colorimetric determination with Nessler's reagent and chloride by titration with standard silver nitrate solution.

TABLE I  
Amino Acids Found in or Recovered from Untreated and Treated 24-Hour Normal Male Urines

Amino acid*	Sample†											
	A-1	B-1	A-1	A-2	B-1	A-1	A-1	B-1	A-1	B-1	A-1	B-1
	Untreated‡						Hydrolyzed with HCl					
	Found						Recovered					
	Hydrolyzed with urease						Hydrolyzed with urease and HCl					
	Found						Recovered					
	Found						Recovered					
mg.	mg.	mg.	mg.	mg.	mg.	per cent	per cent	mg.	per cent	mg.	per cent	
*Arginine	18.8	21.2	29.5	30.2	31.2	95	94	102	30.0	32.6	107	103
Glutamic Acid	0.0	0.0	311	364	425	109	101	104	324	435	89	99
Glycine	632	842	536	362	742	106	108	102	559	702	89	106
*Histidine	166	103	209	189	146	91	98	100	164	140	107	102
*Isoleucine	4.0	2.5	16.2	14.4	15.6	97	132	95	26.1 <sup>3</sup>	25.1 <sup>3</sup>	127	97
*Leucine	11.4	9.8	32.0	27.8	27.1	113	114	123	33.5	32.2	104	111
*Lysine	19.4	18.5	87.0	77.6	71.0	120	119	135	76.1	76.0	125	139
*Methionine	2.3	3.1	13.8	8.6	15.8	75	88	105	12.9	16.1	106	103
*Phenylalanine	10.1	9.4	31.1	25.6	22.9	120	122	143	30.5	29.3	118	122
*Threonine	24.6	22.4	64.2	63.2	65.0	106	113	115	69.5	65.5	104	120
Tyrosine	22.7	13.4	45.7	24.6	38.4	53	76	108	33.9	37.2	118	125
*Valine	6.8	3.2	28.3	26.9	20.7	122	116	137	29.6	25.6	123	114

amino acids could be determined with *L. fermenti* 36 (10, 12), it appears that the urines contained some substances of higher activity than methionine and threonine for *L. arabinosus* 17-5 than for *L. fermenti* 36. This condition was not unexpected in respect to threonine, since it has been observed that pyridoxamine or pyridoxal (14), as well as other amino acids (authors' unpublished data), may replace threonine for the growth of *L. arabinosus* 17-5 but not *L. fermenti* 36.

Since urea is present in urine in high concentration, it seemed probable that this metabolite might be the principal cause of difficulty in assays. For this reason, the effect of removing urea by hydrolysis with HCl, with urease and with both agents was investigated. It appears from the data given in Table I that hydrolysis with urease did not change markedly the quantities of the apparent amino acids present in untreated urine as measured microbiologically. On the other hand, increases in amino acids determined in this manner in urines hydrolyzed with HCl or with HCl and urease, averaged about 300%. The smallest increases, about 50%, were found for arginine and histidine. The results with glutamic acid and glycine were unexpected in that glutamic acid increased from 0 to more than 300 mg. while glycine decreased about 15 per cent.<sup>3</sup> Since acid hydrolysis is convenient and was found to hydrolyze combined amino acids in urine as effectively as treatment with HCl and urease, it was employed in all subsequent work.

Data on the 24-hour urinary excretion of 12 amino acids by 6 normal male and female subjects are given in Table II as the *per cent* of the total amino acids found in each sample. It is to be noted that amino acid excretion on a given dietary regime was nearly constant, even though the urine volume varied from about 900 to as much as 1500 ml. and  $\text{NH}_4^+$  nitrogen from 5 to as much as 11.4 g. The daily excretion of arginine, isoleucine, leucine, methionine, phenylalanine and valine on all diets varied from about 0.5 to 2.5% of the total amino acids determined. The variations ranged from about 2 to about 5% of the total for lysine and threonine, 6-14% for aspartic acid and histidine, 14-

<sup>3</sup> Since the average daily excretion (about 0.7 g.) of hippuric acid is approximately equivalent to the glycine found in the present experiments, it seems evident that a large part of the glycine in unhydrolyzed urines was present as hippuric acid. It is considered probable that the lower values found for glycine in the acid-hydrolyzed urines may be accounted for by hydrolysis of the hippuric acid and by the higher activity (per mole) of hippuric acid than glycine for *L. mesenteroides* P-60 (and several other organisms) (unpublished data obtained by Ruth Malin in the authors' laboratory).

TABLE II  
*Amino Acids Found in Urines Hydrolyzed with HCl*

Amino acid*	Male urine sample†													
	Per cent of total amino acids found in 24-hour urine													
	A-1	A-2	A-3	A-4	A-5	A-6	A-7	A-8	A-9	A-10	A-11	A-12	A-13	A-14
Aspartic acid	11.7	13.5	13.4	13.7	12.1	12.6	8.2	8.4	10.4	9.2	8.4	9.8	9.0	8.8
Arginine	2.0	2.2	2.7	2.6	2.5	2.3	1.8	1.6	1.6	1.8	1.7	2.0	1.9	1.6
Glutamic acid	20.2	26.4	22.8	21.1	22.6	24.8	13.7	14.4	15.0	15.5	14.2	16.6	13.9	14.5
Glycine	34.8	26.3	31.6	30.8	33.1	30.4	55.0	55.8	51.8	50.2	53.4	46.9	52.3	53.0
Histidine	13.6	13.7	11.5	12.9	12.3	12.3	10.4	9.4	10.1	11.1	10.0	11.2	10.4	10.1
Isoleucine	1.0	1.0	1.6	1.4	1.5	1.3	0.8	0.7	0.9	0.8	0.8	0.8	0.9	0.8
Leucine	2.1	2.0	2.2	2.2	2.2	1.9	1.5	1.4	1.6	1.6	1.7	1.8	1.6	1.5
Lysine	5.7	5.7	5.0	6.4	5.4	5.3	2.7	2.7	3.0	3.0	3.2	3.5	3.3	3.2
Methionine	0.9	0.6	0.5	1.0	0.7	0.7	1.0	0.7	0.8	0.8	0.9	1.0	0.9	1.0
Phenylalanine	2.0	1.9	2.2	2.3	2.2	2.2	1.2	1.1	1.2	1.3	1.4	1.5	1.3	1.2
Threonine	4.2	4.6	4.2	3.3	3.3	4.0	2.2	2.3	2.1	3.3	2.7	3.2	3.0	3.0
Valine	1.8	2.0	2.2	2.2	2.1	2.0	1.4	1.3	1.5	1.4	1.6	1.6	1.4	1.4
Mg. of total amino acids	1538	1376	1423	1537	1523	1510	2207	2563	2228	2831	2484	2344	2462	2428
Mg. NH <sub>4</sub> <sup>+</sup> nitrogen in 24-hr. urine	11350	7500	9360	7940	11120	10400	6900	6600	6000	6100	6200	5000	5100	5000
Volume (ml.) of 24-hr. urine	1225	1545	930	880	1190	1200	1400	1230	1330	1235	1605	897	990	1180

TABLE II (continued)  
*Amino Acids Found in Urines Hydrolyzed with HCl*

Amino acid*	Male urine sample <sup>1</sup>					Female urine sample <sup>1</sup>		
	C-1	B-1	B-2	B-3	D-1	E-1	F-1	F-2
	<i>Per cent of total amino acids found in 24-hour urine</i>							
Aspartic acid	8.9	11.3	12.1	11.4	13.2	10.4	7.6	7.2
Arginine	1.8	1.7	1.8	1.9	1.6	1.6	1.5	1.3
Glutamic acid	21.3	23.8	22.7	25.9	19.6	22.8	17.8	17.5
Glycine	42.0	41.6	46.3	41.8	31.5	42.2	52.5	51.8
Histidine	10.4	8.2	5.9	6.7	14.3	9.4	7.4	8.6
Isoleucine	1.3	0.9	0.9	1.0	1.2	1.4	1.0	1.0
Leucine	2.2	1.5	1.3	1.5	2.0	1.6	1.4	1.2
Lysine	4.3	4.0	3.2	3.2	8.3	4.0	5.1	6.3
Methionine	0.6	0.9	0.5	0.8	0.9	0.8	0.7	0.6
Phenylalanine	2.1	1.3	1.5	1.6	2.0	1.7	1.5	1.4
Threonine	3.0	3.6	2.5	2.9	3.6	2.6	2.2	1.9
Valine	2.0	1.2	1.2	1.4	1.8	1.5	1.3	1.2
Mg. of total amino acids	1707	1786	1979	1672	1898	1105	1244	1659
Mg. NH <sub>4</sub> <sup>+</sup> nitrogen in 24-hour urine	9850	5010	8390	8990	11400	6900	6000	7700
Volume (ml.) of 24-hr. urine	850	1248	1480	1200	1230	1120	1200	1405

<sup>1</sup> Samples A-1 to A-6, inclusive, were collected while the subject was on a normal diet. Samples A-7 to A-14, inclusive, were collected while the subject was on a fixed food and water ration (Army K-Ration and 2250 ml. of distilled water). The same quantity of food products, identical in type and composition, was consumed each 24 hours. During the collection of samples A-9 and A-10, the subject ingested 20 gr. of sulfathiazole each day in the treatment of a throat infection which appeared on the third day of the experiment. The age and weight, respectively, of each subject are given in the parentheses: A (24, 174), B (30, 150), C (24, 135), D (24, 170), E (25, 103), and F (55, 114).

\* Aspartic acid was not determined in the preliminary experiments (data summarized in Table I) since the method of Hac and Snell (3) was not available at that time. Tryptophan was not determined since it is destroyed in acid solution. Tyrosine was not determined because of the low recovery found (see Table I) after acid hydrolysis of urines.

26% for glutamic acid and 30-55% for glycine. It is of interest that the percentage values for male and female subjects were approximately the same, even though the total amino acid excretion ranged from 20 to more than 30% lower for the female than the male subjects. That the proportion of glycine was exceptionally high and varied from about 26 to 46% for males on normal diets, 40 to 50% for females on normal diets, and from about 47 to 56% for a male on K-Ration appears to be noteworthy. In all cases the sum of the glutamic acid and the glycine averaged considerably higher than 50% of the total amino acids determined. A summary of these relations and the percentages and weights of 12 amino acids determined in K-Ration are shown in Table III.

TABLE III

*Average Amino Acids Found in Acid Hydrolyzates of Urines  
On Normal Diets and K-Ration. Amino Acids in K-Ration*

Amino acid	24-Hour urine samples						Amino acids ingested as K-Ration	
	All samples <sup>1</sup>		Normal diet samples, A-1 to A-6, Inclusive		K-Ration diet samples, A-7 to A-14, Inclusive			
	Found <sup>2</sup>	Av. <sup>3</sup> dev.	Found <sup>2</sup>	Av. <sup>3</sup> dev.	Found <sup>2</sup>	Av. <sup>3</sup> dev.	Per cent of total determined	Weight (mg.)
Aspartic acid	10.4	14.1	12.8	5.4	9.0	6.5	10.4	7100
Arginine	1.8	6.6	2.4	9.7	1.7	6.8	4.6	3100
Glutamic acid	20.6	10.3	23.0	7.6	14.7	5.0	28.3	19200
Glycine	42.4	9.0	31.2	6.5	52.3	3.8	4.7	3200
Histidine	10.1	19.6	12.7	5.4	10.3	4.2	3.5	2400
Isoleucine	1.1	13.1	1.3	14.4	0.8	5.0	7.5	5100
Leucine	1.7	15.9	2.1	4.7	1.6	5.6	10.8	7300
Lysine	5.0	27.1	5.6	5.9	3.1	7.5	7.9	5400
Methionine	0.8	10.7	0.8	19.3	0.9	8.8	3.0	2000
Phenylalanine	1.7	12.4	2.2	6.5	1.3	9.1	5.9	4000
Threonine	2.9	13.5	3.9	10.6	2.7	14.7	5.5	3700
Valine	1.6	15.6	2.0	6.4	1.5	5.0	7.8	5300
Mg. total amino acids	1684	14.1	1484	3.8	2443	5.8		67800

<sup>1</sup> The samples were those given in Table II for 4 males and 2 females. Each value listed is the average of the mean values for the 6 individuals.

<sup>2</sup> Each value is the *per cent* of the total of the 12 amino acids.

<sup>3</sup> Average *per cent* deviation from the mean.



That there was no close relation between the percentages (of the total amino acids determined) of amino acids in K-Ration and the 24-hour urines is indicated by the significantly higher values for all amino acids except glycine and histidine. The percentage (of the total amino acids determined) of glycine in K-Ration was only about one-sixth to one-tenth of the comparable values for glycine in the 24-hour urines on normal and K-Ration diets.

Nearly all of the urinary amino acids investigated previously have been determined principally by isolation and colorimetric procedures. Many of the literature values have only qualitative significance. Albanese and coworkers have reported that 247-494 mg. of methionine (15), 251-301 mg. of tryptophan (16) and 50-150 mg. of arginine (17) were excreted daily in the urine by normal males on normal diets. It was first found (18) that no isoleucine or hydroxyamino acids could be detected in normal urine but it was reported later (19) that as much as 16 mg. of hydroxyamino acid nitrogen was present in 24-hour specimens. Acid treatment (24-hour hydrolysis with 6 *N* HCl) of 24-hour urines resulted in increases of methionine from 188 to 461 mg., cystine from 49 to 80 mg., hydroxyamino acid nitrogen from 16 to 108 mg., and amino nitrogen from 179 to 588 mg. It was estimated from the latter data that 70% of the total urinary amino acids occur in bound form. The normal values for arginine, histidine and tyrosine were not altered appreciably by acid hydrolysis and the normal value for tryptophan was not changed by alkaline hydrolysis.

The amino acid values reported in this paper are much lower than those found by Albanese *et al.* (15-19). Workers (20-25) who have employed microbiological procedures for the determination of tryptophan, leucine, isoleucine, valine, threonine, lysine, histidine, arginine, phenylalanine and tyrosine in blood, urine and sweat have stated that amino acids were recovered satisfactorily from these fluids, that the values obtained were reproducible and apparently valid, that no inhibitions or stimulations from other substances occurred at the dilutions used, that the methods were considered to have a high degree of specificity and that many of the newer microbiological methods appear to be at least as specific as the chemical ones and much more sensitive. It does not appear impossible, however, that  $\alpha$ -hydroxy acids,  $\alpha$ -keto acids, and perhaps other substances in urine, may simulate amino acids in stimulating or inhibiting growth of microorganisms, and that apparent amino acid values may be too high or too low.

#### SUMMARY

The apparent free and combined forms of 12 amino acids have been determined by microbiological methods in 24-hour urines of normal

male and female subjects on normal diets and K-Ration. It has been found that the excretion of each amino acid was nearly constant on a given dietary regime and that the values were relatively low for arginine, isoleucine, leucine, methionine and phenylalanine. Relatively high percentages were found for glutamic acid and glycine and intermediate values were obtained for lysine, threonine, aspartic acid and histidine. The percentages of glycine and histidine were markedly lower and those of the other amino acids were much higher in the ingested K-Ration than in the 24-hour urines.

Urinary amino acid values obtained by colorimetric and microbiological methods have been compared and discussed.

#### ADDENDUM

Steele *et al.* (26) have found recently that no leucine, lysine, aspartic acid, isoleucine and proline could be detected in some of the urines of female subjects fed eggs or soybeans. Since aspartic acid was found only in acid-hydrolyzed urines, it was suggested that this amino acid "exists in human urine almost entirely in a bound form." Although these workers found from 23 to 90 mg. of free (microbiologically available) glutamic acid in unhydrolyzed urines, no free glutamic acid could be detected under these conditions in the present experiments. Because Steele *et al.* preserved the urines with toluene and HCl, it may be supposed that a small part of the normally-bound glutamic acid may have been hydrolyzed. The inability of these workers to detect leucine, isoleucine and lysine may possibly be explained by the low concentrations of these amino acids in the urine samples assayed. According to the present authors' experience, these amino acids can be determined consistently and satisfactorily only in urines which have been hydrolyzed with acid, distilled to remove the excess acid, compensated to avoid possible effects of the salt on the organisms, and diluted to appropriate volumes (two-fold for leucine and isoleucine and five-fold for lysine).

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# Pectic Enzymes. VIII. A Comparison of Fungal Pectin-methylesterase with that of Higher Plants, Especially Tomatoes\*

R. J. McColloch and Z. I. Kertesz

*From the Biochemical Laboratories of the Division of Food Science and  
Technology of the New York State Agricultural Experiment  
Station, Cornell University, Geneva, N. Y.*

Received October 11, 1946

## INTRODUCTION

In our continued studies of pectic enzymes (1) we had occasion to make a number of observations which might be of interest to other workers in this field. We were specifically interested in determining the behavior of the enzyme pectin-methylesterase (PM) of various origins in order to obtain evidence of the possible existence of different enzymes which might catalyze the deesterification of pectinic acids. The behavior of PM toward chemical inactivating agents has also been of interest to us since the important role of pectic enzymes in determining the consistency of tomato products was noted (2). An obvious manner of counteracting the detrimental action of these enzymes would be by suitable inhibitors. Finally, we were interested in making observations on the PM of fungal origin<sup>1</sup> which occurs in commercial pectinases (3) to determine whether the PM activity in these enzyme preparations might be increased to advantage by the addition of PM of similar nature from higher plants. According to the observations of Jansen, MacDonnell and Jang (4), deesterification will enhance the action of the polygalacturonase (PG). This led to a systematic comparison of the behavior and action of the PM of higher plants, especially tomatoes, and of PM of fungal origin. The results of this study are reported below.

\* Approved by the Director of the New York State Agr. Exp. Station for publication as Journal Paper No. 685.

<sup>1</sup> Pectinol, manufactured by the Rohm and Haas Company of Philadelphia, Pa.

## EXPERIMENTAL

*The pH Optima of Plant and Mold PM*

The pH optimum of the PM of higher plants is highly uncertain. This is due to the effect of cation concentration on the activity-pH relationship and because the activity (under some conditions) increases linearly with increasing pH until the point is reached (around pH 8.0) where alkaline saponification also begins to occur (5). Although the necessity for small amounts of salts (such as NaCl) for the activation of PM has been recognized and employed routinely in laboratories studying PM, it remained for Lineweaver and co-workers (6, 7) to quantitatively evaluate the effect of concentration and kind of cation on the pH-activity relationship of alfalfa and orange PM. They showed that divalent cations had a greater effect than monovalent, and were capable of causing nearly maximum activity to manifest itself over a broad pH range from 4 to 8. The monovalent cations did not produce this latter effect.

Recently Fish and Dustman (3) investigated the enzymes present in Pectinol A and determined the pH optimum of the PM. They found that a definite optimum occurred at pH 4.5 and that the activity declined rapidly toward pH 7.0. Due to the difference between the pH optimum reported by Fish and Dustman for the PM in Pectinol and that found by others for PM from other sources, it seemed advisable to reinvestigate this point. The usual method for the estimation of PM (5) was employed with the slight modifications suggested by Fish and Dustman for determining the activity at low pH values.

The substrate consisted of 50 ml. of 0.5% citrus pectin containing 0.02 *M* NaCl. This mixture in a 250 ml. beaker was mechanically stirred and the extension electrodes of a Beckman pH meter inserted. The mixtures were adjusted to nearly the desired pH, the enzyme solution added and the final pH adjustment made using 0.10 *N* NaOH in all cases. The pH of the reaction mixture was kept constant for 30 minutes with the same base and the mixture then rapidly titrated to pH 7.0. For reactions above pH 7.0 the final titration is neglected since they were still on the steep portion of the neutralization curve of pectinic acids. Blanks were obtained for each enzyme by titrating to pH 7.0 identical mixtures in which the added enzyme had been previously boiled for 5 minutes. For high activities, shorter reaction times were used so that not more than 30% demethylation occurred.

Two Pectinol preparations, "No. 8" and "No. 32," representing alcohol precipitates from extracts of mold cultures were obtained through the cooperation of the Rohm and Haas Company. For the mold PM studies, approximately 2% solutions of these were made up in distilled water, filtered and used immediately. The source of tomato PM was comminuted whole tomatoes passed through a Buchner funnel without paper. An extract of PM was prepared from this slurry by allowing a portion of it to

stand for one hour after adjustment to pH 7.0 with NaOH and filtering on sharkskin paper. Tobacco PM was prepared by shaking 10 g. of powdered tobacco leaves with 100 ml. of water and filtering on sharkskin paper. Lilac PM was obtained by grinding 50 g. of frozen leaves with 100 ml. of water and squeezing through cheesecloth. Alfalfa PM was prepared by comminuting 100 g. of frozen leaves with 200 ml. of water and squeezing through silk. Apple PM was obtained by grinding 25 g. of frozen apple tissue with 26 ml. of water and squeezing through silk.

The dry matter content and pH of the enzyme preparations were determined. All preparations except the apple and tomato were at pH 5.5 or above assuring good extraction of the PM present (8). The activities of the enzymes were determined at pH intervals of about 0.5 units between the range from 4.0 to pH 8.0 and expressed as PMU/g. dry matter (5).

These results are shown graphically in Fig. 1. The superiority of the tomato as a source of PM over the other higher plants tested is noteworthy, although the extraction of PM from the various tissues may not have been complete. MacDonnell, Jansen and Lineweaver (6) have reported a similar activity obtainable from orange flavedo and albedo. The PM from the higher plants investigated shows pH characteristics similar to that of the tomato. There is considerable doubt that the base consumption observed in the case of apple tissue was actually due to the action of PM. The abruptness of the tomato PM curve as compared with the other higher plants is due to the effect of releasing the suppressing action of low pH on large amounts of the enzyme in one case and on small amounts in the other. The increased yield of tomato PM by extraction at an elevated pH is well shown. No explanation is offered for its behavior below pH 4.5.

The observations of Fish and Dustman on Pectinol PM are confirmed, although the pH optima found in this laboratory are slightly higher. This is not inconsistent since the Pectinols used were different and the previous authors had employed several alcohol extractions to remove added substances which were not present in our samples. The pH optimum shown by the Pectinol PM is different enough to suggest that the enzyme here is not identical with that occurring in higher plants. Further evidence of this will be presented below.

#### *Salt Activation of Tomato and Mold PM*

The curves shown in Fig. 1 were obtained with 0.02 M NaCl in the reaction mixture. Inasmuch as the possibility existed that the differences observed in the pH optima might have been due to different

degrees of salt activation, we have determined the shape of these curves with purified enzymes at different NaCl levels.

For some of these tests the highly concentrated tomato PM previously described (1) and showing 200,000 PMU/g. dry matter activity was used. The PM activities were estimated as described on the previous pages with the exception that the enzymes were inactivated before the final titration to pH 7.0 by an agent which will be discussed subsequently. When a salt-free slurry of the purified preparation was tried first, it was found that a time lag occurred before full activity developed. Therefore a dilution of the purified PM containing 117 PMU/ml. in 2.5% NaCl was employed. One-tenth ml. of this solution was used and the amount of NaCl thus

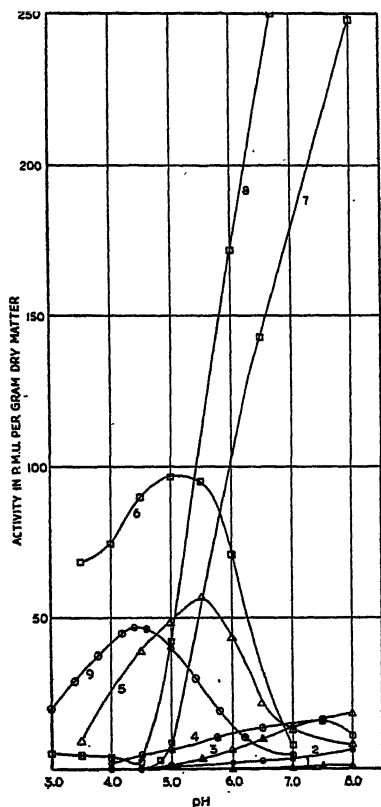


FIG. 1. Activity-pH Relationship of Pectin-methylesterase from Various Sources in the Presence of 0.02 M NaCl at 25°C. Source of enzyme: 1. Apple; 2. Lilac leaves; 3. Alfalfa; 4. Tobacco; 5. "Pectinol 8"; 6. "Pectinol 32"; 7. Tomato ("juice"); 8. Tomato ("extract"). Fish and Dustman's results are shown by curve 9.

introduced considered in adjusting the NaCl molality of the reaction mixtures. The crude tomato PM was dialyzed for two hours to remove most of the salts and 0.5 ml. used in the determination. Any residual amount of salts introduced with the dialyzed enzyme was considered negligible as compared with the experimental molalities.

Some difficulty was encountered in the dialysis of Pectinol due to its destructive action on the viscose membranes employed.

The results of these experiments are shown in Fig. 2. The shapes of the curves obtained are in general agreement with those previously

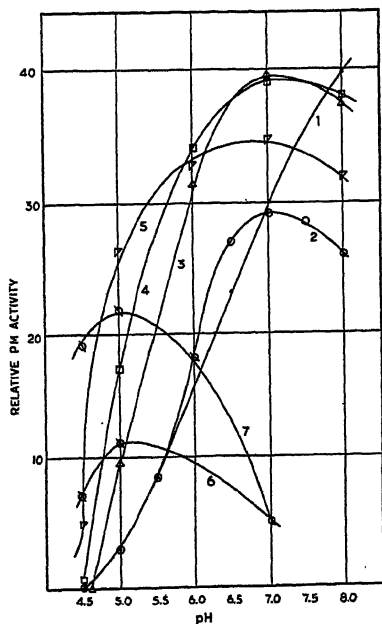


FIG. 2. Effect of NaCl Concentration on Tomato and Mold Pectin-methylesterase Activity. Curves 1 and 2: Crude tomato enzyme with 0.10 and 0.02 *M* NaCl, respectively; 3, 4 and 5: Purified tomato enzyme with 0.02, 0.04, and 0.10 *M* NaCl, respectively; 6: Dialyzed fungal enzyme; 7: Same with 0.10 *M* NaCl.

reported for orange and alfalfa PM (6, 7) and tomato PM (5, 8, 9), strongly indicating the similar nature of the PM from various higher plants. The results with purified tomato PM show two interesting aspects. It appears that the effect of added NaCl on purified tomato PM is enhanced so that small concentrations cause the same type of



activation that larger concentrations achieve for the crude enzyme. Furthermore, higher NaCl concentrations produce the "plateau" effect which was obtained in less purified PM preparations only with the more effective divalent cations such as Ca (6). This suggests that salt activation and its effect on the pH optimum curves of PM may be partly through the influence of salts on substances accompanying PM and not entirely by the mechanism already proposed by Lineweaver and Ballou (7). A different behavior was observed in the case of Pectinol. NaCl activated the dialyzed Pectinol PM but only in the region of its optimum pH (5.0). The differences in the pH optima cannot, therefore, be ascribed to the salts present in the preparations.

#### *Simultaneous Action of Tomato and Mold PM*

It was deemed also possible that a natural non-dialyzable activator or inhibitor in one or the other preparation might account for the observed differences. This possibility was tested in the following experiment.

The pH-activity curves of crude solutions of both enzymes were determined in the usual manner. The amounts of each were chosen so that the same activities were present at their respective pH optima. The activity of each enzyme was separately plotted against pH, and the sum of these activities was also plotted. An experimental curve was then obtained for the activity of a mixture of the above enzymes acting simultaneously in the same reaction mixture at these pH values. The results are shown in Fig. 3.

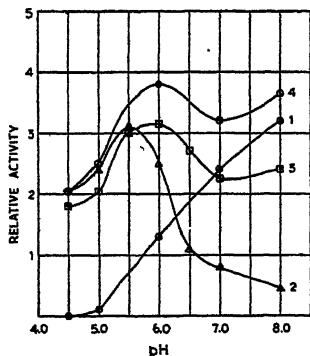


Fig. 3. Simultaneous Activities of Mold and Tomato Pectin-methylesterase in 0.02 *M* NaCl. Curve 1: 1 ml. tomato enzyme; 2: 5 ml. mold enzyme ("Pectinol 32"); 3: 1 ml. tomato and 5 ml. mold enzyme; 4: Sum of activities shown in curves 1 and 2.

Were a specific inhibitor or activator present in either preparation, one would expect, as a result, that the simultaneous action curve would assume predominantly the shape of the curve of the enzyme preparation containing it. Actually, the shape of the curve is nearly identical with that obtained by summing the activities of the enzymes acting independently. The only noticeable effect is an overall suppression of activity which might be expected since the enzymes are compelled to compete for the substrate and, at a low pH, some of the substrate may be combined with relatively inert tomato PM while at a high pH some of it may be combined with relatively inert Pectinol PM. These results strongly suggest that the dissimilarities observed are due to a fundamental difference in the enzymes themselves.

#### *The Effect of Chemical Inactivating Agents*

It has been previously noted (10) that tomato PM is unusually resistant to inhibition by chemical agents other than heavy metal ions. A large number of further agents were tested during the course of this work on both tomato and mold PM. The inhibitory action was tested in the presence of the substrate and under conditions usually employed for the determination of PM. With the tomato PM the following method was used:

Ten ml. portions of 0.5% pectin containing 0.10 *M* NaCl were placed in 50 ml. beakers and a sufficient amount of experimental material added to give a concentration of about 0.50%. A few drops of methyl red and 1 ml. of a crude tomato PM preparation were added, after which the pH was adjusted to about pH 6.5. The mixtures were allowed to stand one hour, keeping the pH constant by adding 0.10 *N* NaOH. Under these conditions complete deesterification occurs in the control mixtures in less than 30 minutes. At the end of the hour, 5 ml. of 2 *M* CaCl<sub>2</sub> were added to each mixture. The formation of a Ca-pectinate or Ca-pectate gel indicated that the pectinic acid had been demethylated by the PM. Failure to form a Ca-pectinate or Ca-pectate gel indicated that PM had not been active.

Those mixtures in which no gel (or only a flocc) was formed upon the addition of CaCl<sub>2</sub> were precipitated by adding two volumes of acid ethanol, filtered on sharkskin filter paper and washed exhaustively with a 2:1 mixture of acid ethanol and water. The precipitate was then washed through the paper with hot water, cooled, and 1 ml. of the same PM added. The mixture was adjusted to pH 6.5 with methyl red. A positive PM reaction (11) indicated the presence of methyl ester groups. This confirms the evidence obtained above that the PM had been inactivated or inhibited.

The literature indicates that CN<sup>-</sup> and I<sub>2</sub> do not inactivate PM (10). The compounds tested in the present experiment were: HgCl<sub>2</sub>, CuSO<sub>4</sub>,

NaCN,  $\text{NaN}_3$ , formaldehyde, benzaldehyde, formic, succinic, phthalic, gallic, picric, iodoacetic and *p*-aminobenzoic acids; chloroform, ethylene chloride, ethylene chlorohydrin, thiourea, phenylthiourea, acetoacetic ester, methyl salicylate, acetyl methylsalicylate, histidine hydrochloride, *m*-phenylenediamine hydrochloride, sodium- $\alpha$ -dinitrophenol, sodium diethyl dithiocarbamate, 2,4-dichlorophenoxyacetic acid (2,4-D), phloroglucinol, neoarsphenamine, alloxan, "Noctal," brucine, piperine, morpholine, pyridine and quinoline.

Of the compounds tested, only pyridine and quinoline demonstrated any tendency to inhibit PM. This effect was then tested under various conditions by the quantitative method. The determination is subject to some uncertainty because the presence of these compounds affects the action of the pH meter and therefore the formation of a Ca-pectinate or Ca-pectate gel was also used as a criterion of PM action. Pyridine and quinoline only inhibited PM at pH 7 or above and at low concentrations of PM in the absence of salts. The action was never completely inhibited if measured by the formation of Ca-pectate after several days. This suggests that the inhibition is of the competitive type and is in accord with the facts that both compounds contain a typical heterocyclic N capable of salt formation with the carboxyl groups of pectinic acid.

Subsequently it was found that a small amount of 10% soap solution completely stopped the action of tomato PM on pectinic acids. A large group of household detergents were then tested and all were found to be more effective than the soap solutions in inactivating PM at all pH values below 8.0.<sup>2</sup> One of the most effective was "Swel" which contains an alkyl aryl sulfonate distributed under the trade name "Nacconol MRSF."<sup>3</sup> The inhibiting effect *in vitro* of detergents on enzyme action has been reported by Freeman *et al.* (12). The inactivation of PM appears to be instantaneous and is practically unaffected by salts or pH. Only at very low concentrations of Nacconol and PM does there seem to be any relationship between the amount of detergent required for inactivation and the PMU present. For example, 11 mg.-% of active ingredient (Nacconol NRSF contains about 90% active material) was found to be the minimum concentration required

<sup>2</sup> In our view, observations of PM activities made above pH 8.0 are unreliable because of concomitant alkaline saponification.

<sup>3</sup> The authors are indebted to the National Aniline Division of the Allied Chemical and Dye Corp., of New York, N. Y., for the Nacconol NRSF.

to quickly inactivate 60 tomato PMU. Doubling the amount of enzyme and then doubling the amount of pectin solution did not cause the resumption of any activity.

We have now adopted Nacconol to improve our method of PM determination. At the end of the reaction period and before the titration is made we add to the reaction mixture 2 ml. of a 1% solution of Nacconol. The enzyme activity which occurs during the back titration is thus eliminated by the inactivation of PM by the Nacconol.

The tests for inactivation of Pectinol PM were necessarily conducted at pH 5.0, the optimum pH for our samples, and only by the titration of liberated carboxyls since the pectinic acid is simultaneously hydrolyzed by PG and will give no Ca-pectate precipitate after the reaction period.

The PM of Pectinols was found to be even more resistant to inactivation by chemical agents than that of tomato fruit. The enzyme is not inactivated by 0.50% concentrations of iodoacetic acid, NaCN, CuSO<sub>4</sub>, I<sub>2</sub>, phenylthiourea, Nembutal, neoarsphenamine, or 2,4-D. More than 2 ml. of saturated HgCl<sub>2</sub> or 40% formaldehyde in the reaction mixture are required for a noticeable reduction of activity.

Pectinol PM is not inactivated by even 100 times the ratio of Nacconol NRSE to PMU which suffices for tomato PM. Near saturation of the reaction mixture with the detergent is required before the mold PM activity falls off at both pH 5.0 and 7.0. Sodium diethyl dithiocarbamate and NaN<sub>3</sub> have an inhibitory effect on the Pectinol PM activity at 0.05 to 0.10% concentrations but they reacted with the substrate as well and the results obtained are somewhat confusing. These agents, however, did not inactivate or inhibit tomato PM at either pH 5.0 or pH 7.0.

Pectinol PM may be removed from its solutions by the proper use of cation exchange resins (13). Tomato PM is not affected by exposure to this ion exchange resin under the conditions which result in the removal of Pectinol PM from its solution.

#### *Observations on the Thermal Behavior of Tomato and Mold PM*

The two enzymes also differ in their relative sensitivity to temperature. Fig. 4 shows the activities of the two enzymes at temperatures from 0° to 50°C. and the effect of holding each enzyme at pH 5.8-6.0 for one hour at elevated temperatures. It can be seen that the PM in

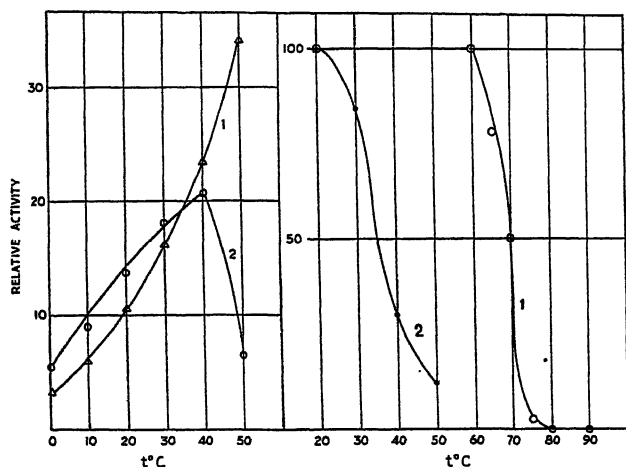


FIG. 4. Left: Effect of Reaction Temperature on the Activities of Tomato (1) and Mold (2) Pectin-methylesterase in 0.10 *M* NaCl. Right: Effect of holding for one hour at various temperatures on the activities of tomato (1) and mold (2) pectin-methylesterase.

Pectinol is rapidly inactivated at 40°C. and above, whereas that from tomatoes is unaffected even at 50°C. Tomato PM is also unaffected by heating at its natural pH of 4.2 for 1 hour at 60°C. (11).

The  $Q_{10}$  values for both enzymes are shown in Table I together with the approximate energies of activation calculated from the Arrhenius

TABLE I  
 $Q_{10}$  and Activation Energies ( $E_a$ ) of Pectinol and  
Tomato Pectin-Methylesterase (PM)

°C.	Tomato PM		Pectinol PM	
	$Q_{10}$	$E_a$	$Q_{10}$	$E_a$
0-10	1.82	9,300	1.67	8,000
10-20	1.80	9,800	1.54	7,200
20-30	1.50	7,300	(1.30)	(4,700)*
30-40	1.44	7,000	(1.11)	(2,100)
40-50	1.46	7,700	(0.31)	—

\* The activation energies of Pectinol PM above 20°C. are meaningless since heat inactivation becomes too rapid.

equation in the usual manner. The tomato PM data are in good agreement with those already published (14).

### DISCUSSION

The pH-activity curves of PM from various sources present no fundamentally new evidence. They do, however, serve to compare their relative activities in a unified assay. The superiority of tomato fruit as a PM source is demonstrated and the observations of Fish and Dustman on mold PM are confirmed.

The effect of purifying tomato PM on its pH-activity curve is shown to be similar to the effect produced by added salts. With such preparations high NaCl concentrations are capable of producing the type of flattened curve which was only obtained with more efficient divalent cations when less pure preparations were used (6).

The differences in the behavior of tomato PM and that produced by molds are striking. They were shown above to differ in their pH-activity relationship, salt activability, in their inactivation by poisons, and in their thermal behavior. Tomato PM is unusually resistant to inactivation by many chemical reagents in small concentrations. From the list of compounds which fail to inactivate this enzyme it would appear that its activity does not directly derive from any of the customary groups present in proteins. It is probable, therefore, that the activity of PM depends on the general physical properties of the surface of the molecule rather than on any functional group. This belief is supported by the fact that the only materials which can truly be said to inactivate the enzyme are surface active agents and that all surface active agents thus far tried show this ability. The fact that the concentration of detergent required is nearly independent of enzyme concentration above a certain minimum further supports the above assumption. It is probable that the inactivation of this enzyme by detergents is due to surface denaturation rather than a combination of the detergent with reactive groups. Anson (15) has shown that such denaturation of proteins may be caused by synthetic detergents.

In addition to the above evidence, it is known that the activity of Pectinol PM is practically unaffected by ethanol precipitation or extraction at pH's from 4.0 to 7.0 (16). On the other hand, tomato PM is very sensitive to ethanol and, once desorbed from tomato pulp, it is readily inactivated by ethanol precipitation (14). Furthermore, dialy-

sis of solutions of tomato PM results in its precipitation without inactivation (1), whereas Pectinol PM remains in solution after dialysis.

The differences shown to exist between the Pectinol PM and that from higher plants, more specifically tomatoes, strongly indicate that the enzymes are fundamentally different. It is interesting to speculate whether this difference may be accompanied by dissimilar mechanisms of action such as the order in which methyl groups are removed.

The indiscriminate use of the PM of higher plants to enhance PG action in commercial pectinases would be of questionable merit. The PM from most higher plants is likely to be inactive at pH 3.5 which is a good average value for the fruit juices in which Pectinol is used. The differences in the thermal properties may also cause difficulties.

#### SUMMARY

1. Pectin-methylesterase (PM) obtained from higher plants show definite similarity in their pH-activity relationship. Experiments are reported to indicate that tomato PM behaves essentially similarly to alfalfa and orange PM studied by other investigators. The PM in Pectinol, produced from mold cultures, has a pH-activity curve different from those of higher plants.

2. The salt activation of purified tomato and Pectinol PM indicated differences in the two enzymes.

3. The two enzymes in the same reaction mixture appear to be able to act independently.

4. The two enzymes also differ in their resistance to chemical inactivating agents, especially detergents.

5. The PM of Pectinol and that of tomatoes also differ in temperature quotients, energies of activation and rates of thermal inactivation.

6. Further differences in the behavior of the two enzymes when exposed to ethanol or during dialysis are stated.

7. The above information is taken as strong evidence that there are at least two enzymes capable of demethylating pectin, one occurring in higher plants and the other in commercial pectinase preparations made from mold cultures.

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# Fibrinogen: With Special Reference to its Preparation and Certain Properties of the Product<sup>1</sup>

Arnold G. Ware, M. Mason Guest and Walter H. Seegers

*From the College of Medicine, Wayne University, Detroit, Michigan*

Received December 9, 1946

## INTRODUCTION

The numerous methods described for the preparation of fibrinogen would seem to preclude the need for any new approaches to the problem. Nevertheless, this important biological product is difficult to purify and the best methods thus far devised yield a product which is defective in one or more respects. Laki (8) obtains crystalline material. The fibrinogen is precipitated with  $(\text{NH}_4)_2\text{SO}_4$ , adsorbed, eluted, and poured into a large volume of warm buffer solution. Crystals soon form. If the crystallization is repeated with bovine fibrinogen denaturation results. This introduces the possibility that the protein is also damaged during the first crystallization.

Cold alcohol fractionation technics used by Cohn and associates (3) fulfill many requirements but leave much to be desired. For example, the painstaking amino acid analyses performed by Brand, Kassell and Saidel (1) involved a product supplied by the Harvard group which was only 87% clottable. Our own use of cold alcohol (15) gave values for purity ranging from 70 to 98%, usually near 85%. Anything better than 85% was largely fortuitous and, even with a product of high purity, the fibrinolytic enzyme was a contaminant, thus yielding an unstable product of limited value. For the operative removal of renal calculi by Dees' procedure (4, 6), fibrinogen which will yield high tensile strength is needed. A special preparation was therefore described by Neurath, Dees and Fox (10) yielding a tensile strength of from 50 to 90 g.

Some of our fibrinogen preparations have a tensile strength in excess of 500 g. They are essentially free of prothrombin, free of the fibrinolytic enzyme and other constituents concerned in the blood coagulation mechanism. The preparations vary only slightly in their reactivity

<sup>1</sup> Aided by a grant from the Research Department of Parke, Davis and Company.

to thrombin from one preparation to another. Solutions are stable for more than 6 months at  $-30^{\circ}\text{C}$ . Upon thawing, the solution is clear and remains so throughout the day, quite unlike other preparations which form variable amounts of precipitate and coagulum. The preparations can be thawed and frozen repeatedly or dried from the frozen state without being denatured. The purity is usually above 95%.

## EXPERIMENTAL

### *Preparation*

The method is based on a simple observation. When fresh frozen plasma is allowed to thaw slowly until all ice is just gone only part of the fibrinogen goes into solution. The fibrinogen in solid phase can be collected by centrifugation, washed, dissolved, and constitutes the new fibrinogen preparation.

Many variations of the general procedure have been tried but only one representative example need be described. At the slaughter house 20 l. of blood were mixed with 1 l. of special anticoagulant (1.85%  $\text{K}_2\text{C}_2\text{O}_4 \cdot 2\text{H}_2\text{O}$  and 0.5%  $\text{H}_2\text{C}_2\text{O}_4 \cdot 2\text{H}_2\text{O}$ ). The oxalated blood was brought to the laboratory and centrifuged. A 1 gallon metal tin can was then filled and frozen solid at  $-30^{\circ}\text{C}$ . This was then placed in an ordinary refrigerator at  $5^{\circ}\text{C}$ . Two days later the plasma contained no ice. Two clean, trunnion cups (International Equipment Co.,  $2\frac{1}{2}''$  i.d.  $\times$   $4\frac{1}{2}''$  deep) were then cooled in an ice bath. Part of the cold plasma was then poured into these and centrifuged at room temperature (2,500 r.p.m. for 1 min.). After decanting this was repeated until all fibrinogen had been collected. This took only a few minutes. The centrifuge cups were again placed in an ice bath. A total of 110 cc. of ice cold saline (0.9% NaCl) was now poured over the fibrinogen. Thorough mixing was then achieved by using a strong glass rod fitted on one end with a No. 7 rubber stopper. This stirrer was used in stomp fashion. A mechanical stirrer denatures. The mixture was then centrifuged at room temperature for 1 min. at 2,500 r.p.m. and, after decantation, the cups were returned to the ice bath. One hundred and ten cc. of ice cold saline were again added and mixed with the fibrinogen. This was again centrifuged for 1 min. at 2,500 r.p.m. The washing was repeated 5 more times with 100, 90, 80, 70, and 50 cc. of ice cold saline. To remove the last 50 cc. of saline 3 min. was allowed for centrifugation. The fibrinogen was then suspended in 200 cc. of saline and set in a water bath at  $35^{\circ}\text{C}$ . The special stirrer was used to keep the mixture in constant motion until the temperature was  $33^{\circ}\text{C}$ . The fibrinogen solution now filled one metal centrifuge cup and was centrifuged for 2 hrs. at room temperature at 2,500 r.p.m. The clear fibrinogen solution was then decanted. The volume was 275 cc. The solution was analyzed by using the technique described below. It was 97.5% clottable with thrombin and represented a 2.6% solution of fibrinogen.

These manipulations require no special equipment, relatively little time, and the molecule is not subjected to any denaturing agents. In fact we consider this material

to be a close approximation to native fibrinogen. This work has been repeated 20 times with such minor variations as we thought might be worth trying. Nevertheless, in 18 cases, the purity ranged from 95.0 to 101.4%, and in the other 2 cases the products were 90.0% clottable with thrombin.

### *Drying*

It has been shown, and we have confirmed, that drying from the frozen state reduces the clot strength of plasma (17). Our fibrinogen preparations have been dried many times. In all instances the material could be dissolved again with mild agitation. Neither the purity, rate of reactivity with thrombin, nor tensile strength was altered by the drying.

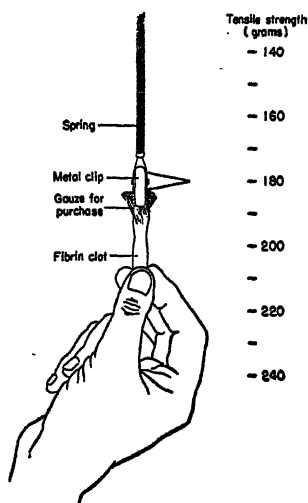


FIG. 1. Arrangement for Measuring Tensile Strength of Fibrin Clots. This is a modification of the Neurath, Dees, and Fox procedure (10).

### *Tensile strength*

Neurath, Dees and Fox (10) clot the fibrinogen in tubes of 8 mm. inside diameter and 50 mm. long. The clot is then removed and one end is held in a clamp. The other end holds a small container. Water is poured into the container until the clot breaks. The weight of the container and water at time of breaking then represents the tensile strength in g. These new preparations can be made to have tensile strengths up to 550 g. and consequently involve considerable splashing of water when the clot breaks. Therefore, we devised the spring system illustrated in Fig. 1. The tensile strength values are the same as with the original method of Neurath *et al.* (10), but we consider this a more efficient arrangement.

The maximum tensile strength of the fibrinogen preparations described is reached within 30 min. after mixing with thrombin (Fig. 2). In that respect the behavior is

similar to the less pure products of Neurath *et al.* (5, 10). For producing the clot thrombin was used (0.2 cc. thrombin + 2 cc. fibrinogen) in sufficient strength to cause clotting in 30 seconds. The fact that these clots maintained their full strength for more than 24 hrs., at room temperature, is interpreted as an indication that they are free of any significant quantity of the fibrinolytic enzyme and this once again shows that thrombin does not itself eventually dissolve a clot as was formerly stated (7, 9, 11).

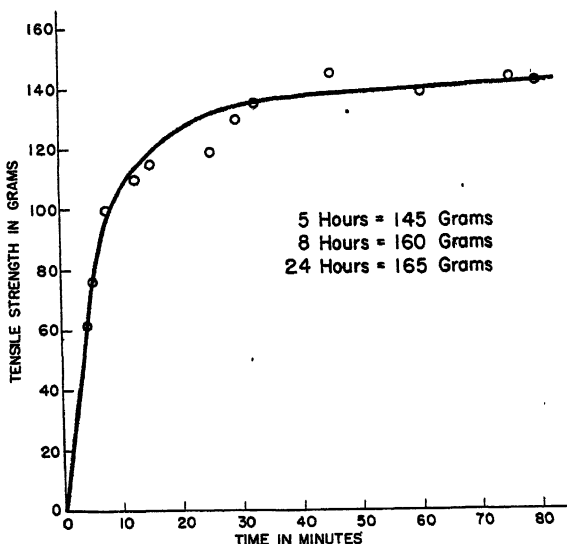


FIG. 2. Development of Tensile Strength in a 2% Fibrin clot, at pH 7.2, Room Temperature (28°C.) and in 0.9% NaCl + 0.1%  $\text{CaCl}_2$  Solution. The clots with thrombin formed in 30 sec. The fibrinogen preparation was 100% clottable with thrombin.

The solubility of fibrin is considerably reduced in the presence of calcium ions (12, 2), and calcium increases the rate at which thrombin clots fibrinogen (13). We have confirmed the fact that the tensile strength of the clot is increased by calcium ions (18). Fig. 3 compares clots produced in 0.9% NaCl with those produced in 0.9% NaCl + 0.10%  $\text{CaCl}_2$ . In addition to this comparison the Figure shows that tensile strength is directly proportional to fibrin concentration. This simple relationship no longer applies, however, to very concentrated solutions of fibrinogen. A 4% solution gave a tensile strength of 550 g. with calcium which is somewhat more than would be predicted from the curve in Fig. 3.

### Clot Retraction

There is no evidence of clot retraction over a period of 24 hrs. provided the clot is stored in an atmosphere saturated with water vapor.

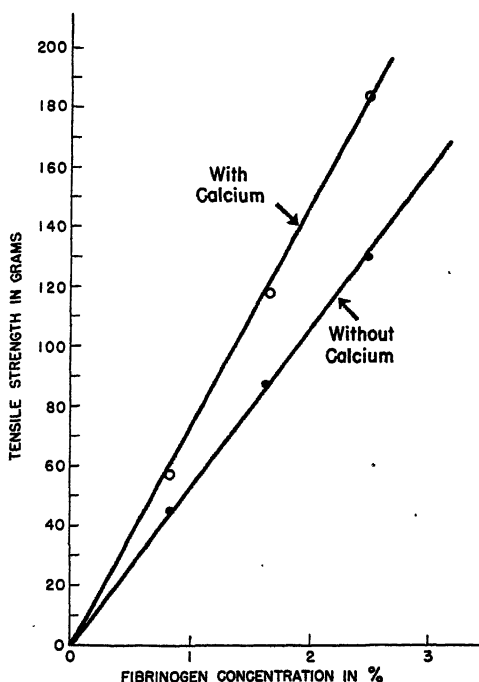


FIG. 3. Tensile Strength of 100% Clottable Fibrinogen in (1) 0.9% NaCl Solution and (2) in 0.9% NaCl + 0.1%  $\text{CaCl}_2$  Solution. The clots with thrombin formed in 30 sec. and measurements were made at 30 min. The pH was 7.2 and the temperature approximately 28°C. Each point on the curve represents an average of 5 separate determinations. The modification of the Neurath, Dees, and Fox method depicted in Fig. 1 was used for the measurements.

### *Analytical*

One cc. of a 0.2–0.8% fibrinogen solution is mixed with 30 cc. of saline containing 1 cc. of  $M/5$  phosphate buffer of pH 6.4 and also 10.5 cc. of 1%  $\text{CaCl}_2$ . The calcium is not precipitated. We prefer to use a 30 × 120 mm. tube of 50 cc. capacity.  $\text{CaCl}_2$  produces a less soluble fibrin, and fibrin formed at pH 6.4 is more easily rolled out than fibrin clots formed in alkaline solution. To the diluted fibrinogen 1 cc. of a 50% glycerol solution (14) containing about 100 Iowa units of commercial thrombin is added. This is then mixed immediately by inversion and allowed to stand 30 min. at room temperature. The clot is then wrapped around a stainless steel wire. The fibrin is removed from the wire and the remaining fluid is expressed mechanically with the aid of filter paper. The fibrin is first washed with tap water and then with 0.9% NaCl. The quantity of fibrin may be determined by measuring the tyrosine or N content.

In the work described above this procedure was used. Tyrosine was measured with the use of Folin-Ciocalteu reagent. The difference between the values before clotting with thrombin and the yield of tyrosine in the clot obtained with thrombin gives the necessary figure for computing *per cent* clottability. We feel that this modification of the original Whipple and Hurwitz (19) procedure has many advantages over numerous modifications which we have tried. Theoretically, one should use highly purified thrombin in place of commercial thrombin in order to avoid possible adsorption of proteins (16) during the clotting process but such a procedure is not generally convenient.

### SUMMARY

When specially oxalated bovine plasma is frozen solid and slowly thawed until no more ice remains, much of the fibrinogen remains in suspension and can be removed by centrifugation. When washed with cold saline and dissolved by warming, a fibrinogen preparation of high purity is obtained. The range of purity is usually from 95–100%. Fibrinogen so prepared has many desirable qualities not possessed by former preparations. Over a wide range, tensile strength is directly proportional to concentration and is increased by calcium ions. A method for measuring fibrinogen concentration quantitatively is described.

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# The Formation of Lactic Acid by *Clostridium acetobutylicum* (Weizmann)

Ernst Simon

*From the Daniel Sieff Research Institute, Rehovoth, Palestine*

Received January 9, 1947

## INTRODUCTION

The diversion of biological processes in an abnormal direction has often been a means of obtaining a deeper insight into their mechanism, especially of establishing the occurrence of intermediate products which normally are transformed too quickly to be isolated. An interesting application of this principle has been made by Kempner (1), Kempner and Kubowitz (2) and Kubowitz (3). They showed that the fermentative production of butyric acid by a strain of *Clostridium butyricum* is diverted into the formation of lactic acid by carbon monoxide. This result, which is the more remarkable as carbon monoxide is without any influence upon the alcoholic fermentation, leads to two conclusions.

(a) In at least one stage of the butyric acid fermentation, a heavy metal or metal complex catalyst is required, for only such catalysts are apt to be inactivated by carbon monoxide.

(b) In the butyric acid fermentation, either lactic acid is itself an intermediate which "normally" is transformed into butyric acid, or there exists an intermediate of such structure that it can either be metabolized to lactic or to butyric acid. It may be noted that Weizmann and Hellinger (4) described a butyric acid producing organism, which normally forms certain quantities of lactic acid.

It seems interesting to investigate whether carbon monoxide also influences the acetone-butyl alcohol fermentation and whether its influence can shed some light on the mechanism of this technically important fermentation.

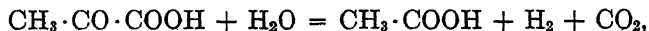
In a previous communication (5) it has been shown that carbon monoxide has no inhibitory effect on the acetone-butyl alcohol fermentation of a 5% maize mash. This statement has been confirmed but



it does not appear to apply to other substrates. The fermentation of a 2.5% maize mash (instead of a 5% mash), of a maize mash saccharified with malt meal, of a malt mash and of glucose-yeast water in the presence or absence of asbestos, is completely inhibited by carbon monoxide. The inhibition is reversible, fermentation becoming normal as soon as the carbon monoxide is displaced, *e.g.*, by nitrogen. It seems as if the different behavior of a 5% maize mash is due rather to mechanical than to any chemical or biochemical factors.

One will conclude again that a factor of heavy metal nature is required by *Cl. acetobutylicum*: the cells do not multiply in a freshly prepared mash when carbon monoxide is present from the beginning. If, on the other hand, the bacterial cells are isolated from a fermenting mash after 24 hours, centrifuged and washed, they do ferment glucose in an atmosphere of carbon monoxide. The fermentation leads, however, under these conditions, not to acetone and butyl alcohol, but exclusively to racemic lactic acid. Under these conditions, both the number and the enzymatic activity of the cells is greater than in a freshly inoculated mash.

Obviously, the butyric acid and the acetone-butyl alcohol fermentation have similar mechanisms. In neither case is it possible to say as yet, where the carbon monoxide interferes with the normal metabolism of the bacteria. One will have to study the influence of that gas on each stage of the fermentation. A first step in this direction has been made by the investigation of the fermentation of pyruvic acid with *Cl. acetobutylicum* in an atmosphere of carbon monoxide. Even under normal conditions, pyruvic acid is atypically fermented, giving only acetic acid. In presence of carbon monoxide, the bulk (86%) remains unaffected by a centrifuged and washed preparation of *Cl. acetobutylicum*. It appears possible that the enzyme normally responsible for the conversion of pyruvic acid into acetic acid, presumably by the mechanism



is sensitive to carbon monoxide and, therefore, involves a heavy metal.

It is interesting to note that Lipman (6, 7) has found the enzyme in *B. delbrueckii* which converts pyruvic to acetic acid, requires manganese or cobalt. In his case, the hydrogen set free was transferred to another molecule of pyruvic acid, giving lactic acid (See Still (8)).

There is a second means of diverting the acetone-butyl alcohol fermentation into the formation of lactic acid. It has been known that hexose-1,6-diphosphate (Harden-

Young ester) is converted by *Cl. acetobutylicum* to methylglyoxal only (9). In presence of glutathione, the specific coferment of glyoxalase (10, 11), lactic acid is formed instead in quantitative yield. In this respect, *Cl. acetobutylicum* shows a close resemblance to yeast for which the same phenomenon has been described by Auhagen and Neuberg (12).

### EXPERIMENTAL

#### *I. Influence of Carbon Monoxide on the Normal Fermentation with Cl. acetobutylicum*

Exp. No.	Substrate
1 and 2	1 l. 5% maize mash, inoculated with 100 ml. 5% fermenting maize mash.
3 and 4	1 l. 2.5% maize mash + 17 g. glucose, inoculated with 100 ml. 2.5% fermenting maize mash.
5 and 6	1 l. 5% saccharified maize mash, inoculated with 100 ml. 5% fermenting saccharified maize mash.
7 and 8	1 l. 5% malt mash, inoculated with 100 ml. 5% fermenting malt mash.
9 and 10	1 l. yeast water, containing 2% glucose, inoculated with 100 ml. fermenting 2% glucose-yeast water.
11 and 12	1 l. yeast water, containing 2% glucose + 4 g. asbestos, inoculated with 100 ml. fermenting 2% glucose-yeast water.

In experiments No. 1, 3, 5, 7, 9 and 11 from the moment of inoculation a continuous stream of carbon monoxide was passed through the medium for 24 hours. The duplicate experiments were normal fermentations.

#### *Distillation of the Resulting Liquors*

Exp. No.	1	2	3	4	5	6	7	8	9	10	11	12
ml. Neutral solvents	15	16	1	15.5	1	17	0.5	13	1	7.2	2	7.1

Apart from the case of 5% maize mash (Exp. No. 1) all substrates are inhibited by carbon monoxide. This inhibition is reversible.

#### *Reversibility of the Carbon Monoxide Inhibition*

Exp. No.	Substrate
1 and 2	1 l. 2.5% maize mash + 17 g. glucose, inoculated with 100 ml. fermenting 2.5% maize mash.
3 and 4	1 l. 2% glucose, containing 0.05% yeast autolysate + 0.07% asparagine + 4 g. asbestos, inoculated with 100 ml. fermenting glucose solution.

After inoculation, a continuous stream of carbon monoxide was passed through the medium for 24 hours. No fermentation occurred. Nitrogen was then passed through the medium for 15 minutes. After several hours' incubation, normal fermentation set in, and normal amounts of the fermentation products were obtained.

## *II. Influence of Carbon Monoxide on the Fermentation with Centrifuged and Washed Bacteria*

*Bacterial Culture.* 1 l. 5% maize mash was inoculated with 100 ml. fermenting 5% maize mash. After 23 hours of vigorous fermentation, the bacteria were centrifuged, washed several times with physiological salt solution, and the residue was suspended in 250 ml. water.

*Fermentation:* Through 1 l. medium, containing 0.3% glucose, 335 ml. *M/15* phosphate buffer (pH 6.2) and the 250 ml. bacterial suspension, a continuous stream of carbon monoxide was passed. After 18 hours at 37°C., the sugar had completely disappeared.

*Isolation of Lactic Acid.* The fermentation mixture was made alkaline, heated to boiling and centrifuged. The residue was washed several times with 0.85% salt solution and the combined extracts evaporated on the water bath in the presence of potassium carbonate. After acidification with phosphoric acid, the sirupy residue was mixed with anhydrous sodium sulphate and subsequently extracted with ether in a Soxhlet apparatus. After removal of the ether, the liquid was boiled with zinc oxide and some water until neutral, filtered and evaporated. The residue was taken up with water, treated with charcoal and evaporated again, until crystallization set in. Yield of zinc lactate 1.36 g. (Anal.: Calc. for  $C_6H_{10}O_6Zn + 3H_2O$ ; ZnO, 27.4;  $H_2O$ , 18.2; Found: ZnO, 27.7, 27.7;  $H_2O$ , 18.5, 18.9).

The number of hydrate water molecules showed that racemic zinc lactate was formed, this being confirmed by polarimetric investigation.

## *III. Influence of Carbon Monoxide on the Fermentation of Pyruvic Acid with Centrifuged Bacteria*

*Bacterial Culture.* 5 l. 1% maize mash, enriched with 0.1% yeast extract, were inoculated with 250 ml. fermenting 1% maize mash. After 20 hours fermentation, the bacteria were centrifuged, washed with 0.85% NaCl solution, and suspended in 200 ml. water.

### *Fermentation.*

1. 100 ml. 1.33% neutralized pyruvic acid solution
- 200 ml. *M/15* phosphate buffer (pH 6.2)
- 170 ml. bacterial suspension
- 530 ml. water

A continuous stream of carbon monoxide was passed through the medium for 24 hours.

2. 10 ml. 1.33% neutralized pyruvic acid solution  
20 ml. *M*/15 phosphate buffer (pH 6.2)  
20 ml. bacterial suspension  
50 ml. water

Time (hours)	Exp. 1	Pyruvic acid (mg.)	Exp. 2
0	12.05		11.99
24	10.38		0

The pyruvic acid was determined as the 2,4-dinitrophenylhydrazone. For the analysis, 5 ml. 10% trichloroacetic acid solution were added to 15 ml. of the reaction mixture. The precipitate was filtered and made up with water to 25 ml., 15 ml. filtrate being used for each determination.

*Result.* Carbon monoxide inhibits the fermentation of pyruvic acid. In the absence of carbon monoxide, the only product formed is acetic acid (Duclaux analysis).

#### IV. Experiments with Hexose-1,6-Diphosphate

- Exp. 1: 13.3 ml. sodium hexose-diphosphate solution (corresp. to 565 mg. free acid).  
20 ml. *M*/15 phosphate buffer, 20 ml. bacterial suspension, 46.7 ml.  $H_2O$ .  
Exp. 2: As 1, with addition of 20 mg. glutathione.  
Exp. 3: As 1, with addition of 100 mg. iodoacetic acid.  
Exp. 4: 10 ml. 3% glucose, 20 ml. *M*/15 phosphate buffer, 20 ml. bacterial suspension, 50 ml.  $H_2O$ .  
Exp. 5: 10 ml. 3% neutralized pyruvic acid solution, 20 ml. *M*/15 phosphate buffer, 20 ml. bacterial suspension, 50 ml.  $H_2O$ .

After 5 hours' incubation at 37°C., there was no appreciable attack on glucose (Exp. 4) and pyruvic acid (Exp. 5), whereas in the samples with hexose-diphosphate after deproteinization and addition of 2,4-dinitrophenylhydrazine, methylglyoxal was precipitated. Glutathione and iodoacetic acid had no influence under these conditions.

After 24 hours' incubation, glucose and pyruvic acid had completely disappeared. Methylglyoxal was present in the experiments with hexose-diphosphate as substrate.

To investigate the influence of glutathione, the following series of experiments were made:

- Exp. 1: 1.5 ml. sodium hexose-diphosphate solution, 2.0 ml. *M*/15 phosphate buffer, 2.0 ml. bacterial suspension, 4.5 ml. water.

Exp. No.	2	3	4	5	6	7	8	9
As 1, but with addition of:	2 mg.	5 mg.	10 mg.	20 mg.	10 mg.	20 mg.	50 mg.	100 mg.
		glutathione				iodoacetic acid		

After 5 hours' incubation at 37°C., methylglyoxal was found in *all* experiments, but in Exp. 5 only in traces

After 24 hours' incubation, in Exp. 5 no methylglyoxal was present, whereas in all other experiments the characteristic yellow-orange precipitate was obtained on addition of dinitrophenylhydrazine. Five ml. of reaction mixture from Exp. 5 were deproteinized by phosphotungstic acid, treated with copper-chalk solution and filtered. The filtrate was used for lactic acid estimation. Consumption of *N*/100 iodine in Exp. 1: 0.275 ml.; in Exp. 5: 1.05 ml. 4.32 mg. lactic acid had formed, which is 13% of theory, calculated on hexose-diphosphate employed. The hydrazones obtained in the other experiments were filtered off, washed with hydrochloric acid and water, and 10% sodium carbonate solution was added. The *brown* filtrate indicated the presence of *pyruvic acid*. Result: Within 5 hours, hexose-diphosphate is decomposed with formation of methylglyoxal and traces of pyruvic acid. In the presence of *glutathione* (0.3 mol/mol hexose-diphosphate), methylglyoxal is replaced by lactic acid. Iodoacetic acid does not inhibit the transformation of hexose-diphosphate.

#### SUMMARY

Lactic acid, which, under normal conditions, is not a metabolic product of the acetone-butyl alcohol fermentation, is produced by *Clostridium acetobutylicum* under two conditions:

- (a) in an atmosphere of carbon monoxide, if a centrifuged and washed preparation of the bacterium is used;
- (b) from hexose-diphosphate in the presence of glutathione.

It can be concluded that a heavy metal or heavy metal complex is a necessary factor for the transformation of an as yet undefined intermediate which can either give lactic acid or acetone and butyl alcohol.

Pyruvic acid, which is normally transformed into acetic acid by *Cl. acetobutylicum*, remains unaffected by a centrifuged and washed preparation of the bacterium in an atmosphere of carbon monoxide.

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# Some Investigations on the Decrease in Ascorbic Acid Content of Untreated Dehydrated Vegetables During Storage

Eigil Ettrup Petersen and Fritz Schønheyder

*From the Chemical Laboratory, Atlas Ltd., Copenhagen, and the Biochemical Institute, Aarhus University, Denmark*

Received January 13, 1947

It is generally stated that a decrease in the reducing substances, of which ascorbic acid is the most important, occurs during storage of dried vegetables. Apparently no authors have studied the kinetics of the decomposition of ascorbic acid during storage, wherefore this question has been made the subject of closer examination in what follows.

All authors agree that dried vegetables lose ascorbic acid quickly during storage and that the loss increases at rising temperature (8, 9, 11). Aykroyd (1) examined the loss of ascorbic acid at 37°C. in dried, steam blanched vegetables and found that generally about 50% of the vitamin was decomposed during 12 weeks. In dried potatoes, however, the vitamin showed a somewhat greater stability (10% loss during 12 weeks). Laursen and Orth (8) have tried to record the loss curve for vitamin C in a series of vegetables during 9 months, in the course of which period almost all the vitamin C was decomposed. Their curves are difficult to treat mathematically, as the loss during the drying process is included in the curves and the authors do not publish the individual experimental results. The data given by Chace (4), by Beardsley, Prindle and Stevens (2) and by Malette, Dawson, Nelson and Gortner (10) are also too inadequate to give a clear picture of the kinetics of the vitamin decrease in dehydrated stored vegetables.

In the following pages it will be shown that, when the temperature is kept constant and the water contents only vary within rather narrow limits, the logarithm of the ascorbic acid ( $y$ ) in dried vegetables will vary linearly with the storage time ( $t$ ). The loss of ascorbic acid thus proceeds according to a first order reaction scheme. Consequently, the decrease in ascorbic acid content can be characterized by the time of half decomposition ( $t_{0.5}$ ). The half decomposition time of ascorbic acid in kale, white cabbage and spinach, under identical conditions, will be



mentioned. The relationship between  $t_{0.5}$  of ascorbic acid and the water contents of the materials in the three vegetables mentioned is further elucidated. Some of the experiments in this paper have previously been published in Danish in the dissertation of one of us (7).

### MATERIALS AND METHODS

The fresh vegetables are carefully washed in cold water, minced and dried in a current of air at 90°C. in the case of kale and spinach and at 60°C. in the case of white cabbage. The dried materials are homogenized and placed in desiccators over a mixture of saturated salt solution and salt in substance in a thermostat at 25°C. At suitable intervals samples are removed for the determination of ascorbic acid and dehydroascorbic acid. The amounts of these acids are given as mg./100 g. dry matter. The determination of ascorbic acid is carried out according to the method of Birch, Harris and Ray (3). Dehydroascorbic acid is determined by the method of Emmeri and Eekelen (5). The inaccuracy of the determination of ascorbic and dehydroascorbic acid is about 2%. That the reduction value found is due to ascorbic acid has been checked on a sample of spinach. By titration, the sample was found to contain 35 units = 1.75 mg. ascorbic acid/g. of substance. A biological determination on the sample, performed in the State Vitamin Laboratory in Copenhagen, showed that the same material contained more than 25 units/g. but less than 35 units/g.

*Determination of Water Content.* In the case of spinach and kale a weighed amount of material is placed in the thermostat at 105°C. After standing at 105°C. for 24 hours, constant weight was reached. White cabbage was placed on a heating-plate at 90°C. *in vacuo* until constant weight was reached. This special procedure in the case of white cabbage is chosen because the sugar content of the material is caramelized at the temperature of 105°C.

*Desiccators.* The salts employed to keep the desired vapor pressure in the different desiccators are given in Table I. Equilibrium occurred slowly in desiccators 1, 7 and 8.

TABLE I  
*List of Substances Employed in the Desiccators*

Desiccator No.	Salt
1	"Silica gel" in substance
2	Calcium chloride in substance
3	Potassium acetate in sat. solution
4	Potassium carbonate in sat. solution
5	Calcium nitrate in sat. solution
6	Sodium nitrite in sat. solution
7	Potassium nitrate+ammonium chloride in sat. solution
8	Ammonium chloride in sat. solution

### EXPERIMENTAL

The investigation has been performed on the dried products of kale, white cabbage and spinach. In cases where the value of the reducing substance is falling rapidly, as, for instance, in white cabbage, the

TABLE II  
*Experiments on the Loss of Ascorbic Acid in Dried Kale*

Des. No. 1				Des. No. 2				Des. No. 3			
Days	W Per cent	A mg.-%	A+D mg.-%	Days	W Per cent	A mg.-%	A+D mg.-%	Days	W Per cent	A mg.-%	A+D mg.-%
0	(6.2)	752		0	(4.5)	373	380	0	(4.5)	373	380
27	(2.0)	605		10	4.4	377		10	5.2	340	
54	(2.0)	573		22	4.7	357		22	4.3	328	340
82	2.5	500		31	3.6	336		31	6.2	315	
105	3.0	496		40	4.2	292		40	(5.4)	272	
126	2.6	490		48	5.0	306		48	6.2	297	
147	2.2	455		54	4.3	292		54	5.5	274	
207	2.2	420		60	2.9	287	302	60	4.5	271	273
345	2.2	333									
499	2.0	268									
Des. No. 4				Des. No. 5				Des. No. 6			
Days	W Per cent	A mg.-%	A+D mg.-%	Days	W Per cent	A mg.-%	A+D mg.-%	Days	W Per cent	A mg.-%	A+D mg.-%
0	(4.5)	373	380	0	(4.5)	373	380	0	(4.5)	373	380
9	(4.8)	365		9	(6.5)	362		6	(8.8)	353	
19	7.2	333		19	8.3	316	334	14	12.5	272	
28	7.7	316	319	28	8.5	279	400	20	13.1	188	218
38	7.4	260		38	8.5	244		24	12.7	162	
48	7.5	243		48	8.5	203		29	13.2	151	
52	6.2	232		52	8.7	193		34	11.5	120	
59	7.5	229	240	59	9.2	183	187	39	12.6	106	
								45	12.9	86	
Des. No. 7				Summary of the Experiment							
Days	W Per cent	A mg.-%	A+D mg.-%	Desiccator No.	$\alpha_w$	$-b$	$b.s$				
0	(4.5)	373	380	1	2.4	0.0006773	444				
6	(9.8)	359		2	4.2	0.0024643	122				
14	13.5	205	197	3	5.4	0.0020384	148				
20	14.5	165	158	4	7.2	0.0044758	67				
24	14.3	133	139	5	8.6	0.0062415	48				
29	16.0	116		6	12.6	0.015103	20				
34	14.5	79		7	15.0	0.018855	16				
39	15.8	68									
45	16.8	57									

determinations are carried out at 5–6 day intervals. At greater stability the intervals are prolonged.

In the following, results for kale are recorded in tabular form and the data on white cabbage and spinach are reproduced as curves. For the 3 vegetables examined a summary of the half decomposition times in relation to the water contents is given in curves.

*Kale.* In Table II are incorporated all the results of analyses for the materials of kale from the same experiment, kept in 7 different desiccators. As seen from the Table, single determinations of ascorbic acid + dehydroascorbic acid have been carried out. It is seen that there is no indication that dehydroascorbic acid is accumulated in the materials during storage. The same was found to be true in the case of dried white cabbage and spinach. Dehydroascorbic acid, accordingly, cannot be supposed to decompose more slowly than ascorbic acid. The dehydroascorbic acid determinations are too few to be made the subject of a mathematical treatment. In all experiments the dehydroascorbic acid only comprises a small part of the total ascorbic acid.

For the different materials, kept in separate desiccators, an analysis of the regression of the log of ascorbic acid content on experimental time has been carried out. The regression lines are, with sufficient accuracy, straight lines, and the inclination of the straight line, the regression coefficient,  $-b$ , has been computed according to the method of least squares. The values given in brackets in Table II are not included in the statistical treatment. The half decomposition time,  $t_{0.5}$ , results from  $0.3010/-b$ . The values of  $-b$ ,  $t_{0.5}$  and the average water contents  $a_w$  in the different experiments with kale appear in Table II.

*White Cabbage and Spinach.* The results from a series of experiments on the same materials in white cabbage and spinach are recorded in Fig. 1 and Fig. 2 (ordinate: log of ascorbic acid content in mg.-%; abscissa: duration of storage in days).

It is seen that in some experiments the linear relationship between the log of ascorbic acid content and the time includes about 85% of the vitamin C destruction.

In Fig. 3 the times of half decomposition, calculated from the different experiments, are plotted in relation to the water contents of the materials. Apparently the stability of ascorbic acid is greater at low water content. It decreases rapidly with increasing water content and ultimately reaches a very low value at high water content in the materials.

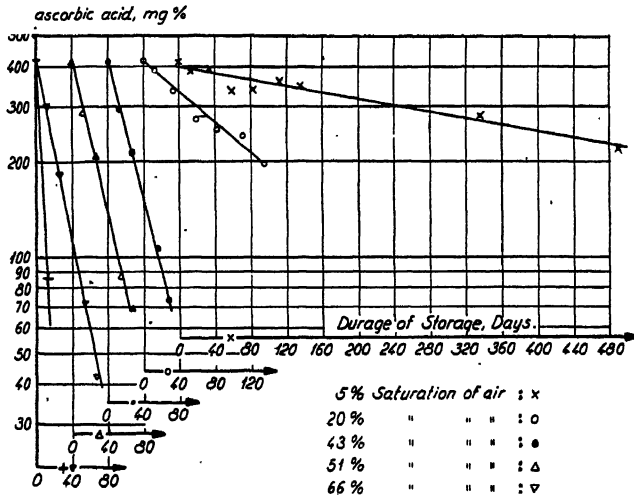


FIG. 1. Decrease of Reduction Value in Air-Dried White Cabbage at 25°C. and Varied Air-Saturation.

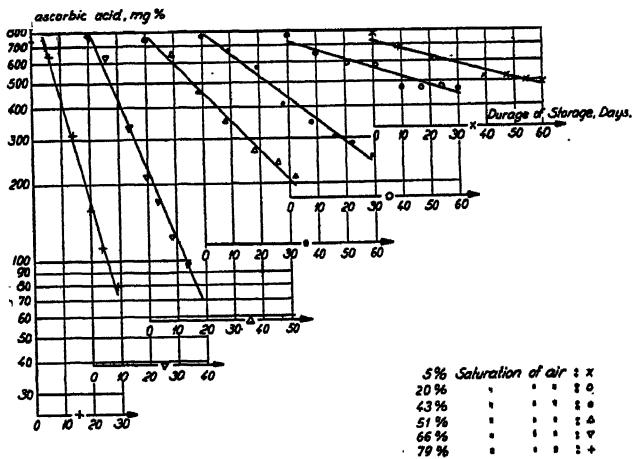


FIG. 2. Decrease of Reduction Value in Air-Dried Spinach at 25°C. and Varied Air Saturation.

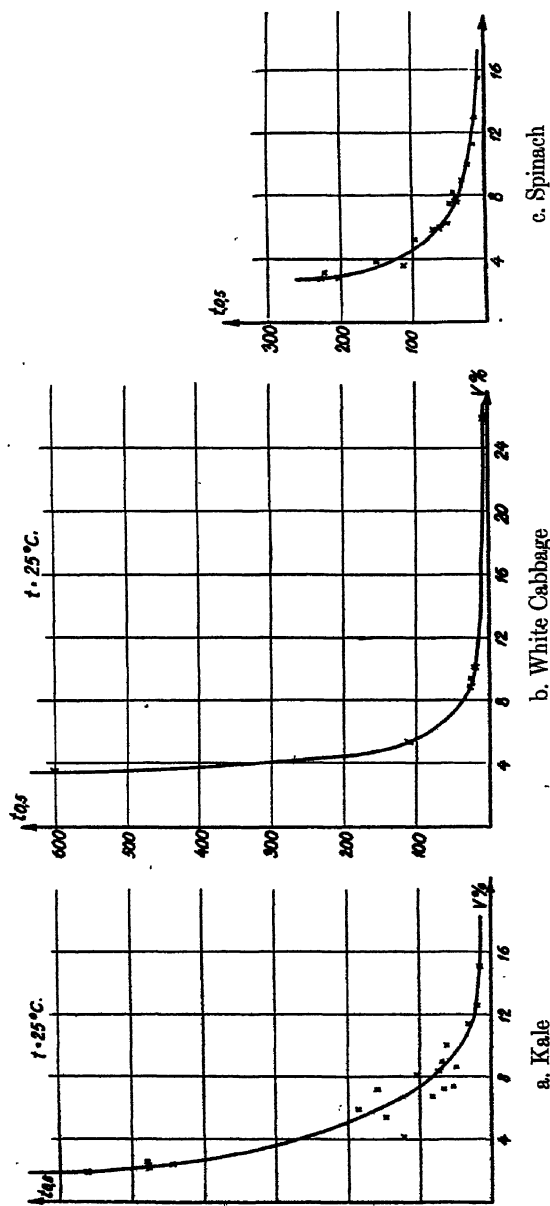


Fig. 3. Influence of Water Contents in Air-Dried Vegetables on the Half Decomposition Time of Their Ascorbic Acid Contents,  $t = 25^\circ\text{C}$ .

## DISCUSSION

It appears from the experiments carried out, that in dehydrated vegetables stored under constant external conditions (temperature and degree of humidity) there is a linear relationship between the log of the reducing values of the materials, calculated as ascorbic acid, and the duration of storage. The oxidation proceeds according to a first order reaction scheme, and it is therefore justifiable to characterize the decomposition of ascorbic acid by the time of half decomposition. Only the first part of the process, apparently, does not satisfy the equation of the monomolecular reaction. The reason for this is presumably that at this time humidity equilibrium has not been attained. The observation that the stability of vitamins is dependent upon the moisture content of dehydrated vegetables is not new. Thus Chace (4) and others have obtained the same result, but it seems to us that the experiments as carried out by us, must be of great practical importance, because they facilitate the statistical treatment of the storage experiments and indicate a practical method of evaluating the stability of ascorbic acid in dried materials under different conditions. The experiments reported show that the ascorbic acid in stored materials of kale, white cabbage and spinach is practically stable at very low humidity. Malette, Dawson, Nelson and Gortner (10) have found a correlation between the ascorbic acid content and storage deterioration (abnormal color and odor development). Our experiments have verified this observation.

In connection with our results it is of interest to mention that Ettrup Petersen and Schønheyder (6) have found that the carotene loss in dried vegetables also proceeds according to a first order reaction scheme. But whereas there is no doubt that the ascorbic acid is most stable at high contents of dry matter the contrary is valid in the case of carotene. In dried carrots we thus found that at a water content of about 18% the stability of the carotene was maximal and decreased with water contents both above and below 18%. The optimal water content for the stability of carotene is certainly different in the different types of dehydrated vegetables. The ideal conditions for storage with regard to ascorbic acid and carotene in different vegetables are therefore very different.

## ACKNOWLEDGMENT

The authors are indebted to cand. act. Th. Busk for the statistical treatment of the results.

## SUMMARY

In dehydrated kale, white cabbage and spinach, stored at constant temperature and moisture, it is shown that the ascorbic acid loss proceeds according to a first order reaction scheme. Hence, it is rational to calculate  $t_{0.5}$  and use this as a measure of the stability for the vitamin mentioned. A close relationship is found between  $t_{0.5}$  and moisture. The stability of ascorbic acid is very great at low water content. It decreases rapidly with increasing water content and is very low at high water content in the stored dried vegetables.

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# The Electrophoretic Analysis of Snake Venoms

J. Moura Gonçalves<sup>1</sup> and Alfred Polson<sup>2</sup>

*From the Laboratory of Physical Chemistry, University of Wisconsin, Madison*

Received February 17, 1947

## INTRODUCTION

Valuable information regarding the protein composition of snake venoms can be obtained by electrophoresis. Polson, Joubert and Haig (1), in their studies of the electrophoretic composition of cobra venom, have shown that the hemolytic and neurotoxic activities are both associated with the same component in the electrophoretic diagram.

In the following communication an account is given of the electrophoretic composition of the venoms of *Crotalus terrificus* (Argentina) and *Bothrops jararacussu* (Brazil). These venoms were presented to the authors by the Bacteriological Institute in Buenos Aires and the Biological Institute of Minas Gerais.

## EXPERIMENTAL

The electrophoresis experiments were carried out in a modified Tiselius apparatus with a long, single cell of 11 ml. capacity (2). A pH 7.7 phosphate buffer of  $\mu = 0.1$  and a pH 8.6 diethylbarbiturate-citrate buffer of  $\mu = 0.088$  were used in most of these experiments. Electrophoretic samples were prepared by 24-hour dialysis at 1°C. of a concentrated solution of the venom against a large volume of the buffer used. Just prior to the experiment the venom was diluted to a protein concentration of 3% with the dialyzate. The conductivity of the dialyzate was measured in the usual manner. A constant potential gradient and a temperature of 1°C. were maintained during the experiment. The duration of electrophoresis varied from 2 to 4 hours, the experiment being discontinued when resolution of the components was satisfactory. The moving boundaries were photographed by using the cylindrical lens schlieren method in conjunction with a diagonal knife edge. After enlargement, projection, and tracing of the photograph the areas were measured with a planimeter and the percentage composition was calculated in the usual arbitrary manner on both the descending and ascending sides and the symmetry determined from these data.

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<sup>1</sup> Fellow of the Rockefeller Foundation.

Permanent Address: Institute of Biophysics, University of Brazil, Rio de Janeiro.

<sup>2</sup> Onderstepoort (South Africa) Laboratories.



The components concerned with toxicity and proteolytic activity were located by withdrawal of samples after suitable electrophoretic resolution. This was accomplished as follows. The electrophoretic columns were cut off in the usual way and the water level in the thermostat lowered below the top of the cell. All electrophoretic equipment accessories were removed and the electrophoretic columns were again connected. Samples were then removed by careful pipetting with a capillary needle, the point of which was bent at a right angle. The pipetting was followed by visual observation to determine which components were being removed. Such removals were carried out on both ascending and descending limbs and from 9-12 samples taken for any given experiment.

Toxicity was determined by the effect produced on intraperitoneal injection into mice of dilutions of the various samples. The proteolytic activity was determined by the tyrosine method of Anson (3), using denatured hemoglobin as the enzyme substrate. From such measurements for samples removed from the electrophoretic cell on both ascending and descending limbs the maximum activity as associated with a given component is easily determined.

## EXPERIMENTAL RESULTS

### *Crotalus terrificus*

Electrophoretic diagrams of the venom of this animal in pH 7.7 and pH 8.6 buffers are shown in Figs. 1A and 1B, respectively. It is evident from these diagrams that no symmetry exists between the descending and ascending boundaries. In the phosphate buffer, 30% of the protein, as indicated by the photographic areas, is migrating anodically in the ascending and approximately 58% in the descending side. In the diethylbarbiturate-citrate buffer the percentage of ascending and

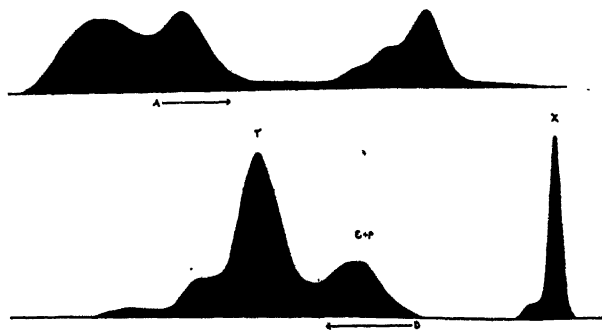


FIG. 1A. Venom of *Crotalus terrificus*. Phosphate buffer,  $\mu = 0.1$ , pH 7.7, T 10,800 seconds, potential gradient 3.4 volts/cm.

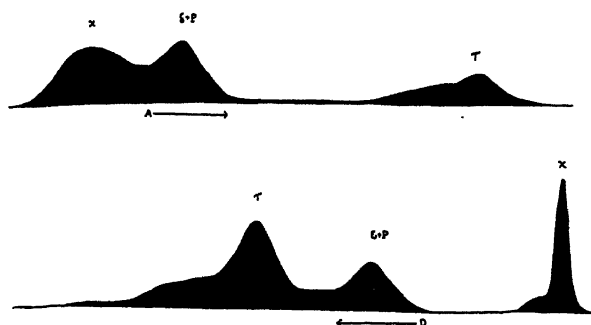


FIG. 1B. Venom of *Crotalus terrificus*. Diethylbarbiturate-citrate buffer,  $\mu = 0.088$ , pH 8.6,  $T = 200$  seconds, potential gradient 8.6 volts/cm.

descending areas showing anodic migration was 21% to 44%, respectively. The presence of relatively large amounts of protein trapped in the salt boundaries in both of these experiments is evident. It is readily recognized that the symmetry failure is related to the presence of the component of high isoelectric point, which is designated as "X". In the above electrophoretic experiments it is evident that there is complex formation taking place between positively and negatively charged protein ions.

To minimize the protein-protein interaction, an electrophoretic experiment at pH 8.6 and protein concentration 1.8% was carried out

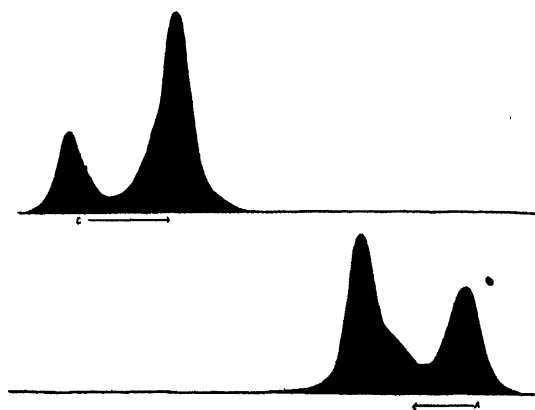


FIG. 1C. Venom of *Crotalus terrificus*. Diethylbarbiturate-citrate buffer,  $\mu = 0.3$ , pH 8.6,  $T = 12,600$  seconds, protein concentration 1.8%, potential gradient 1.6 volt/cm.

at an ionic strength of 0.3. The electrophoretic diagram pictured in Fig. 1C shows a good deal of symmetry as regards ascending and descending sides, the area ratios of the patterns for these sides being 0.83. However, as would be expected, the best results concerning symmetry of the two electrophoretic limbs were obtained in a buffer system of pH 3.0,  $\mu = 0.1$ , (0.5 *N* NaCl, 0.05 *N* glycine hydrochloride).

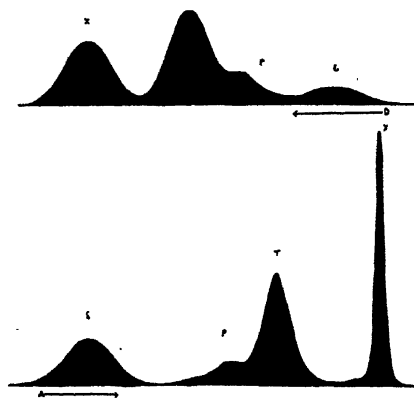


Fig. 1D. Venom of *Crotalus terrificus*. Glycine buffer  $\mu = 0.1$ , pH 3.0,  $T = 14,400$  seconds, potential gradient 3.4 volts/cm.

All components possess the same charge in this buffer and the interaction experienced at higher pH values is eliminated, as shown in Fig. 1D. The *per cent* composition of the various components is shown in Table I.

TABLE I  
*Electrophoretic Analysis of Snake Venoms*

	1		2		3		4		5		Symmetry
	<i>m</i>	<i>per cent</i>	<i>m</i>	<i>per cent</i>	<i>m</i>	<i>per cent</i>	<i>m</i>	<i>per cent</i>	<i>m</i>	<i>per cent</i>	
<i>Crotalus terrificus</i>	3.2	4.26	4.75	10.4	7.12	45	9.6	1.83	12.3	38.4	0.90
<i>Bothrops jararacussu</i>	6.25	67.8	8.9	7.42	10.15	24.8	—	—	—	—	

Composition *per cent* in glycine buffer pH 3.0, ionic strength 0.1.

The mobilities are expressed in  $\text{cm}^2/\text{volt sec.} \times 10^5$ .

The toxicity was found to be associated with the component designated as *T*. Proteolytic activity was found in a component that could not be resolved away from the salt boundaries (Figs. 1A and B). Mouse toxicity tests with material obtained from veronal buffer experiments were performed after removal of the barbiturate by dialysis.

*Bothrops Jararacussu*

Electrophoretic experiments with solutions of the venom of *Bothrops jararacussu* in pH 7.7 phosphate buffer showed a marked difference in electrophoretic pattern (Fig. 2A) as compared with that seen for *Crotalus terrificus*. Mouse toxicity was associated with the component designated as "T" while the proteolytic activity went with a component

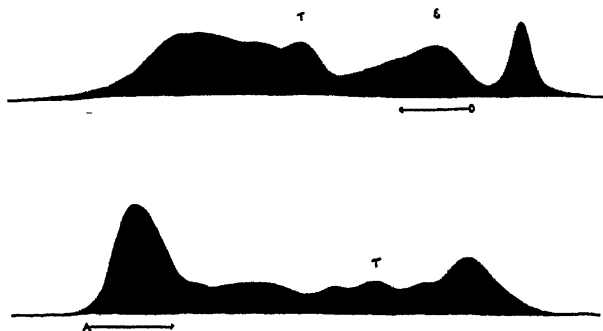


FIG. 2A. Venom of *Bothrops jararacussu*. Phosphate buffer  $\mu = 0.1$ , pH 7.7,  $T = 10,800$  seconds, potential gradient 6.6 volts/cm.

of slightly slower mobility than "T". Due to the better resolution experienced on the ascending side, the biological results on samples removed by pipetting were more clear cut here than on the descending side. Marked asymmetry as regards ascending and descending patterns are evident from an inspection of Fig. 2A, and were not corrected appreciably by raising the ionic strength to 0.2. However, as was true in the case of *Crotalus terrificus* venom, the asymmetries were largely eliminated in pH 3.0 glycine buffer. From Fig. 2B it can be seen that the major portion of the protein migrates as a broad heterogeneous area. In pH 10.0 glycine buffer, and at a protein concentration of 1.8%, the resolution was still poor for this venom. At this pH the component

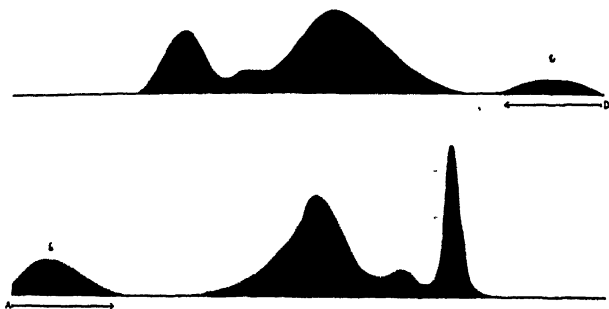


FIG. 2B. Venom of *Bothrops jararacussu*. Glycine buffer  $\mu = 0.1$ , pH 3.0,  $T = 14,400$  seconds, potential gradient 3.4 volts/cm.

designated as "X" is close to its isoelectric point and remains in the salt boundary.

From the electrophoretic analysis of the two venoms studied in pH 7.7 phosphate and pH 8.6 diethylbarbiturate-citrate buffers a marked lack of symmetry is noticed between the components and their areas. Such asymmetry can be explained by the interaction of proteins carrying opposite charges at that pH which results in the formation of protein-protein complexes, the extent of which decreases with low protein concentration and high ionic strength, as found for experiments with *Crotalus terrificus* venom. When all constituents carried charges of the same sign, as is true in pH 3.0 glycine, the interaction largely disappeared. This situation may be compared to the case of the reaction between ovalbumin and ovomucoid as observed electrophoretically by Longworth, Cannan and MacInnes (2). The electrophoretic interaction observed for the venom of the rattlesnake from Argentina (*Crotalus terrificus*) does not appear in the venoms of *Bothrops jararacussu*, a reptile found in the center and north of Brazil. The venoms of the latter snakes do not possess the X component (4).

The location and assignment of the biological activities to certain proteins of a given snake venom will make easier the concentration of these constituents by fractionation. Further, any information about the location of the isoelectric points of the several proteins that a snake venom may possess will permit one to approach the problem of the fractional separation of a given component with a greater opportunity for success. The preparation of an anti-serum to the toxic

factors of given venom may likewise be facilitated if the antigen represents a single entity of the venom.

#### ACKNOWLEDGMENTS

The authors are indebted to Drs. H. F. Deutsch and J. W. Williams of this Laboratory for their aid and advice in the development of the project.

#### SUMMARY

Venoms of *Crotalus terrificus* (Argentina) and *Bothrops jararacussu* (Brazil) were analyzed electrophoretically in buffers at various pH's and ionic strengths.

The toxic and proteolytic constituents were located by biological tests with components which had been removed by a pipette after electrophoretic resolution of the venom.

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# Maintenance of Liver Glycogen by Rats Fasted after Feeding Individual Amino Acids\*

W. R. Todd, Joan M. Barnes and Lew Cunningham

*From the Department of Biochemistry, University of Oregon  
Medical School, Portland*

Received February 17, 1947

## INTRODUCTION

In 1938 Mirski and coworkers (1) reported that "the carbohydrate reserves of the liver laid down on a protein diet behave differently from those deposited on a diet of carbohydrate." In unfasted animals they demonstrated a higher average liver glycogen in rats maintained on a carbohydrate diet. However, following a 24-hour fast, or exposure to cold for several hours, the protein (meat or casein) fed animals invariably showed glycogen reserves higher than those that had consumed the carbohydrate diet.

In work experiments it was found that carbohydrate-fed animals had higher liver glycogen levels immediately after the work, but that the rate of recovery, *i.e.*, glyconeogenesis, was far more rapid in the animals that had consumed the high protein ration.

This capacity of protein to maintain carbohydrate reserves was termed the "protein effect." These authors found that if a high protein diet was fed for as little as one day the "protein effect" could be demonstrated. They reported that 50% protein was the minimum and 70% protein the optimum level for demonstrating this effect. It was established that total adrenalectomy abolished the "protein effect." The main thesis of Mirski (1) in regard to the "protein effect" has been amply confirmed by Newburger and Brown (2), Guest (3), and others.

The purpose of the present work is to establish the extent to which individual amino acids, fed in conjunction with an otherwise satisfactory synthetic diet, are capable of affecting the maintenance of carbohydrate reserves in the rat during a 24-hour fast.

\* Aided by grants from the General Research Council, Oregon State System of Higher Education, and the Diabetic Research Foundation of Portland, Oregon.



## EXPERIMENTAL

*Rations.* The control ration had the following composition in percentage: casein, 16; Wesson salt mixture (4), 5; Brewer's yeast (Squibb), 10; cod liver oil, 2; Wesson oil, 5; commercial white corn dextrin, 54; and dextrose, 8.

The experimental rations differed only in that the desired amino acid was substituted for an equal weight of dextrin. Thus, a 10% glycine diet refers to one containing only 44% dextrin and 10% glycine, with the other constituents unchanged.

Food intake was equalized among experimental and control animals. The amount eaten was 12 or 15 g./rat/day.

*Animals.* Male albino rats of the Sprague-Dawley strain (approximately 200-300 g.) were used throughout. Only data from animals that consumed the designated amount of ration were used.

*Methods.* Liver glycogen was determined by the method of Good, Kramer, and Somogyi (5). Results are expressed as *per cent* of wet weight. Sufficient data have been obtained to indicate that the water content of livers from animals on the various diets is constant within very narrow limits. Fuhrman and Field (6) have reported that the water content of liver is independent of the glycogen level.

*Plan of a Typical Experiment.* Rats were removed from the stock diet (Purina Dog Chow) and placed on the control diet for 24 hours. This procedure accustoms the animals to synthetic rations and leads to better food consumption. The animals were then fed the experimental diet for 48 hours, or left on the control diet for this period. To control the length of the fasting period a portion of the ration (4-6 g.) was withheld until the last two hours of the 48-hour feeding period. After zero, or 24 hours fasting, the animals were sacrificed for liver glycogen determinations. Nembutal anesthesia was used routinely.

## RESULTS

Rats maintained on a 70% protein (66% casein, 10% yeast containing 40% protein) diet show, after a 24-hour fast, about 1% liver glycogen. Mirski's (1) work in this regard is thus again confirmed.

Pertinent data on amino acid feeding in relation to maintenance of liver glycogen are found in Table I. Either 10 or 15% glycine in the diet invariably brings about a marked "protein effect." A 12% DL-alanine diet shows no effect, while at a level of 18% this amino acid elicits a mild response.

At lower levels—5% glycine or 6% DL-alanine—no effect could be demonstrated. It was also impossible to demonstrate any increased carbohydrate storage during a 24-hour fast as the result of feeding L-glutamic acid or L-leucine at levels equimolecular with 10% glycine. These data are not included in the table.

The data indicate that the zero hour fasting glycogen levels are higher following a 12% DL-alanine diet than after a 10% glycine diet, whereas the reverse is true in animals fasted 24-hours after consuming

TABLE I  
*Typical Liver Glycogen Levels of Rats Prefed Diets  
 48 Hours, Fasted 0 or 24 Hours*

Diet fed	Mean liver glycogen (per cent wet weight)	
	Hours fasted	
	0	24
Control	3.9 (14)	0.33 (30)
10% glycine	4.2 ( 8)	1.10 (21)
15% glycine	3.1 (13)	1.17 (16)
12% DL-alanine*	5.0 ( 8)	0.36 (23)
18% DL-alanine*	3.0 (14)	0.56 (22)

\* 12 and 18% DL-alanine are approximately equimolecular with 10 and 15% glycine respectively. Figures in parentheses represent number of animals.

these diets. In fact, inspection shows no relation between zero and 24 hour fasting liver glycogen levels.

### DISCUSSION

The "protein effect" must be accounted for by mechanisms more subtle than simply a constant rate of glycogen withdrawal during fasting. This is evident from the fact that with the diets studied no correlation exists between zero and 24 hour fasting liver glycogen levels.

There is also no correlation between the total nitrogen of the diets studied and their "protein effect."

The accepted glycogenetic properties of the amino acids studied do not account for the results obtained. The amino acids DL-alanine and L-glutamic acid are known glucose and glycogen formers. Glutamic acid exerts no "protein effect" at the levels studied. Twelve *per cent* DL-alanine produces no "protein effect" and 18% DL-alanine only a slight effect.

Recently Olsen, Hemingway, and Nier (7) fed mice glycine containing C<sup>13</sup> in the carboxyl group. They reported that during the 16 hours after feeding, about 50% of the tagged carbon atoms appeared in the respired CO<sub>2</sub> and that only a small part of the carbon of the ingested glycine occurred in the isolated liver glycogen. These results were interpreted as an indication of a promotion of glyconeogenesis by glycine rather than a direct conversion of this amino acid to glycogen. These authors considered that their data upheld Dakin's (8) earlier view on

this point. The data reported herein can also be interpreted as evidence for stimulation of glycconeogenesis by glycine, and assumes additional significance in view of the growing feeling that glycine itself may not be converted into glycogen in the rat.

Another explanation of the "protein effect" of glycine involves the possible glycostatic capacity of the acid. At present sufficient data are not at hand to indicate whether increased glycconeogenesis, decreased glycolysis, or both, account for the "protein effect" of glycine.

As might be expected, the "protein effect" of glycine cannot be demonstrated in adrenalectomized rats.

### SUMMARY

A marked effect on carbohydrate storage in rats during starvation has been demonstrated as a result of prefeeding glycine.

After feeding a diet containing 10 or 15% glycine (replacing an equal weight of carbohydrate in the control ration) for 48 hours, the animals have liver glycogen levels of over 1% at the end of an ensuing 24-hour fast. Under like conditions, rats on the control ration have liver glycogen levels that average around 0.3%. This phenomenon is referred to as the "protein effect."

The amino acids L-leucine and L-glutamic acid do not show this effect when fed at levels equimolecular with 10% glycine. At this same level DL-alanine shows no effect. At a level equimolecular with 15% glycine, however, DL-alanine shows a slight effect.

Adrenalectomized rats exhibit no "protein effect" as the result of prefeeding glycine.

It is felt that sufficient data are not available to state whether the "protein effect" of glycine is due to increased glycconeogenesis, decreased glycolysis, or both.

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# The Beneficial Effects of Synthetic Pteroylglutamic Acid on Lactation<sup>1</sup>

Marjorie M. Nelson<sup>2</sup> and Herbert M. Evans

*From the Institute of Experimental Biology, University of California, Berkeley*

Received February 24, 1947

## INTRODUCTION

Long-term generation studies by Nelson and Evans (1) resulted in the formulation of improved purified diets which fulfilled all the dietary requirements for growth of weanling rats and for reproduction in both sexes. Lactation, although greatly improved, was not optimal. By using a short-term procedure of three weeks, it was found that several B vitamin concentrates furnished varying amounts of the missing factor(s) necessary for optimal lactation (Nelson and Evans (2)). The greatest improvement in lactation was obtained from a liver eluate powder which possessed a high level of the *L. casei* factor. This improvement was in marked contrast to the lack of beneficial effects on lactation of low levels of a "folic acid" concentrate (Nelson, van Nouhuys and Evans, (3)). Similarly, in preliminary reports Cerecedo<sup>3</sup> and Vinson (4) observed beneficial effects of "folic acid" concentrates on lactation while Richardson and Hogan (5) found no correlation between the "vitamin Bc" activity of liver concentrates and their beneficial effects on lactation.

This study reports the beneficial effects of high levels of synthetic pteroylglutamic acid<sup>4</sup> on lactation and on the maternal leucocytes at

<sup>1</sup> Aided by grants from the Board of Research and from the Department of Agriculture of the University of California, and the Rockefeller Foundation, New York City. The following materials were generously contributed: crystalline B vitamins and  $\alpha$ -tocopherol from Merck and Company, Inc., Rahway, New Jersey; synthetic pteroylglutamic acid (Liver *L. casei* factor) and liver fraction powder (Lab. No. 7-5293) from Lederle Laboratories, Inc., Pearl River, New York.

<sup>2</sup> General Mills Fellow.

<sup>3</sup> Cerecedo and Mirone (33) have recently reported improved lactation in mice supplemented with high levels of the synthetic *L. casei* factor.

<sup>4</sup> Synthetic pteroylglutamic acid (Liver *L. casei* factor) was generously supplied by Lederle Laboratories, Inc., through the courtesy of Dr. T. H. Jukes.

weaning. Levels of the liver eluate powder containing similar amounts of pteroylglutamic acid result in considerably greater improvement and thus show that an unknown factor(s) present in the liver powder is necessary for the rat during this period.

### EXPERIMENTAL PROCEDURE

The short-term procedure is the same as previously described (2). Adult stock animals of the Long-Evans strain, together with their litters, were placed on the experimental diets at parturition. The litters were limited to 6 young, preferably 3 males and 3 females. All animals, mother and young, were weighed every five days and the young were weaned on the 21st day following parturition. Diet was given *ad libitum* and the food intake was measured.

The basal purified diet 836<sup>5</sup> was used throughout this study. All vitamin supplements were incorporated in the diet by substitution for an equivalent amount of sucrose. The stock diet (1) plus lettuce *ad libitum* was used for normal controls.

Total and differential blood counts were carried out by standard procedures at the time of weaning (Day 21). Bureau of Standards certified pipettes, Trenner or Thoma type, were used. Two to three hundred cells, usually the latter number, were counted on the Wright-stained slide for the differential count. Blood samples were taken at a standard time (2 PM  $\pm$  30 min.).

As previously reported (3) blood counts were carried out on many rats early in lactation (Day 5). These may be summarized as follows: 12 rats maintained on the stock Diet I + lettuce averaged 12,360 leucocytes (range 7,300–21,000), 69%, or 8,740, lymphocytes and monocytes (range 4,965–16,590) and 31%, or 3,630, granulocytes (range 1,710–4,945). Seventy-four rats placed on various purified diets at parturition averaged on the fifth day of lactation 12,060 leucocytes (6,150–20,000), 70%, or 8,650, lymphocytes and monocytes (4,025–17,400) and 30%, or 3,500, granulocytes (1,250–6,690). These values show that the first 5 days of lactation on a purified diet do not affect the white blood cells and give additional evidence for the normal range of white blood cell types (see Table I). Since the maternal leucocytes, at weaning for animals maintained on the stock diet show no significant change from the blood counts taken the fifth day of lactation,<sup>6</sup> the Day 5 blood count has been elim-

<sup>5</sup> The high-carbohydrate diet 836 consists of alcohol-extracted casein 24%, sucrose 64%, hydrogenated vegetable oil (Crisco) 8%, and salts No. 4 (6) 4%. Crystalline vitamins are added per kg. diet: 2-methyl-1,4-naphthoquinone 5 mg., thiamine HCl 5 mg., riboflavin 10 mg., pyridoxine HCl 5 mg., *p*-aminobenzoic acid 10 mg., nicotinic acid 20 mg., calcium pantothenate 50 mg., inositol 400 mg., and choline chloride 1 g. One cc. of a fat-soluble vitamin mixture containing 6 mg.  $\alpha$ -tocopherol (Merck), 115 chick units vitamin D, 800 U. S. P. units vitamin A and 650 mg. corn oil (Mazola) is given weekly to each litter. The addition of 2-methyl-1,4-naphthoquinone (Menadi-one) is the only change in the diet as previously reported (2).

<sup>6</sup> A lactation leucopenia under different experimental conditions has been reported for stock rats (Emmel, Weatherford and Streicher (7)) and for mice (Gardner and Dougherty (8)). In both studies a proportional decrease of lymphocytes and neu-

TABLE I

*Lactation of Stock Animals Maintained on Diet I + Lettuce ad Libitum*

Series No.	No. of litters	Av. weaning wt. young†	Wt. change of mother during lactation*	Maternal leucocytes at weaning		
				Total WBC* /mm <sup>3</sup>	Lymphocytes and monocytes	PMN
1	10	g. 53 51	g. +22.6 <sup>10</sup> (-3 to +62)			
2	15	50 47	+22.6 <sup>11</sup> (-5 to +57)			
3	12	53 51	+14.5 <sup>11</sup> ( 0 to +28)	12,375 <sup>12</sup> (7,350-17,300)	9,035 (4,770-14,530) 72%	3,340 (2,350-4,720) 28%
4	10	48 49	+22.0 <sup>9</sup> (-1 to +66)	11,145 <sup>9</sup> (6,550-16,750)	7,730 (5,045-10,310) 70%	3,415 (1,365-6,700) 30%
5	11	48 44	+21.5 <sup>11</sup> (-1 to +59)	10,180 <sup>12</sup> (6,700-16,200)	7,150 (3,700-10,660) 70%	3,030 (1,775-5,670) 30%
6	12	49 46	+15.4 <sup>12</sup> (+4 to +33)	10,045 <sup>12</sup> (7,350-15,250)	7,035 (5,220-10,370) 70%	3,010 (1,260-5,150) 30%
Average	70	50 48	+19.6 <sup>64</sup> (-5 to +66)	10,850 <sup>43</sup> (6,550-17,300)	7,675 (3,700-14,530) 70%	3,175 (1,260-6,700) 30%

\* Superscript numbers in this and the following tables indicate the number of animals on which the average is based. Only mothers weaning 5-6 young are included in the columns on weight change and on maternal leucocytes.

† In this and the following tables the upper figure in this column refers to the average weaning weight of male rats and the lower figure to the average weaning weight for female rats.

trophiles was observed. Both groups of investigators removed the young from the nursing mother part of the time. In addition, Emmel *et al.* removed the food and carried out the blood counts at different hours of the day. The importance of a uniform time for taking blood samples has been pointed out by Sabin *et al.* (9).

inated and only the Day 21 counts are reported in the following tables. The data for lactation and maternal leucocytes are given for each series of litters maintained on the stock diet (Table I) and on the basal purified diet 836 (Table II) to show the extent of the variation to be expected in experimental groups. In these two tables the series (each series consists of 10-12 litters) are arranged chronologically, starting with the earliest experiments. The first two series on the stock diet were carried out simultaneously with the first two groups on the purified diet. Thereafter, one or two litters on each diet were started for every week that vitamin supplements were being tested.

TABLE II

*Lactation of Stock Animals Placed on the Basal Purified Diet (836) at Parturition*

Series No.	No. of litters	Av. weaning wt. young	Wt. change of mother during lactation	Maternal leucocytes at weaning		
				Total WBC /mm <sup>3</sup> .	Lymphocytes and monocytes	PMN
1	9	g. 41 40	g. -28.1 <sup>8</sup> (-5 to -54)			
2	9	45 43	-23.3 <sup>9</sup> (-6 to -52)			
3	12	44 41	-21.1 <sup>10</sup> (-6 to -46)			
4	9	42 40	-30.0 <sup>9</sup> (-2 to -76)	4,160 <sup>12</sup> (2,625-6,500)	3,455 (2,510-5,520) 85%	705 (60-2,730) 15%
5	12	40 38	-23.6 <sup>11</sup> (+2 to -52)	4,590 <sup>11</sup> (1,800-6,600)	3,545 (1,780-4,725) 79%	1,045 (20-2,770) 21%
6	9	40 39	-36.9 <sup>9</sup> (+1 to -68)	4,355 <sup>9</sup> (1,650-7,500)	3,575 (1,320-5,560) 85%	780 (95-2,250) 15%
7	10	42 41	-19.3 <sup>9</sup> (+1 to -41)	4,300 <sup>10</sup> (1,550-7,300)	3,375 (1,550-4,890) 81%	925 (0-2,410) 19%
Average	70	42 40	-26.0 <sup>86</sup> (+2 to -76)	4,350 <sup>42</sup> (1,550-7,500)	3,485 (1,320-5,560) 82%	865 (0-2,770) 18%

Two or three series (*i.e.*, 20-30 litters) are considered necessary for significant results, although the data from a single series are highly indicative.

### RESULTS

On the stock diet (Table I) the lactating mothers gain approximately 20 g. in body weight and wean young averaging 48 g. (females) to 50 g. (males). At weaning the maternal leucocytes (43 rats) average 10,850 cells of which 70%, or 7,675, are lymphocytes and monocytes and 30%, or 3,175, are granulocytes (PMN). The granulocytes show a remarkable consistency in percentage and absolute number (30% and more than 3,000 cells) from series to series while the non-granular cells exhibit more variation.

In contrast, the lactating mothers placed on the purified diet (Table II) lose approximately 26 g. in body weight and wean young averaging 40 g. (females) to 42 g. (males). At weaning the maternal leucocytes (42 rats) average 4,350 cells of which 82%, or 3,485, are lymphocytes and 18%, or 865, are granulocytes. While the average values for white blood cells, both total and differential counts, are consistent from series to series, there is considerable variation among the rats in a single series as can be seen by the ranges (given in parentheses). Such variability is to be expected if age, body weight, and/or intestinal synthesis are concerned in the requirement for the missing factor(s). In every series, one or possibly two rats will exhibit a blood count normal in every respect (although in the lowest part of the normal range). However, 86% of the counts fall below the normal range for total leucocytes (*i.e.*, below 6,000) and 74% are below the normal minimum of granulocytes (*i.e.*, 1,250). The drop to pathological levels of granulocytes (*i.e.*, less than 750 cells) occurred in 60% of the group.

Mothers placed on the purified diet averaged 186 days of age and 272 g. in body weight at parturition. They weaned 95% of their young and consumed 27.8 g. of food daily. The corresponding values for rats maintained on the stock diet are: 180 days of age, 275 g. in body weight, 96% of their young weaned, and 36.3 g. of food consumed daily.

#### *Synthetic Pteroylglutamic Acid*

The striking improvement resulting from the addition of various levels of this synthetic vitamin (10) is shown in Table III. Two series of litters were used for each level. The weaning weights of the young are



TABLE III

*Effect of Synthetic Pteroylglutamic Acid and Liver Eluate Powder on Lactation and Maternal Leucocytes*

Supplement per 100 gms. Diet 836	No. of litters	Av. weaning wt. young	Wt. change of mother during lactation	Maternal Leucocytes at Weaning		
				Total WBC /mm <sup>3</sup> .	Lymphocytes and monocytes	PMN
None	70	$\frac{g.}{42}$ 40	$\frac{g.}{-26.0^{65}}$ (-76 to +2)	4,350 <sup>42</sup> (1,550-7,500)	3,485 (1,320-5,560) 82%	865 (0-2,770) 18%
110 $\gamma$ P. G. A.*	26	$\frac{42}{42}$	+ 8.6 <sup>11</sup> (-26 to +35)	7,545 <sup>23</sup> (2,700-13,725)	5,325 (1,780-9,785) 68%	2,220 ( 920-4,255) 32%
275 $\gamma$ P. G. A.*	23	$\frac{46}{43}$	+ 6.3 <sup>22</sup> (-19 to +43)	7,650 <sup>20</sup> (4,200-12,900)	5,565 (2,445-9,160) 71%	2,085 ( 880-4,130) 29%
550 $\gamma$ P. G. A.*	24	$\frac{46}{44}$	+ 7.1 <sup>13</sup> (-14 to +32)	7,300 <sup>23</sup> (3,350-16,600)	5,100 (2,550-11,400) 71%	2,200 ( 270-5,955) 29%
0.2% Liver† =110 $\gamma$ P. G. A.	35	$\frac{47}{46}$	+ 2.5 <sup>33</sup> (-21 to +26)	7,995 <sup>21</sup> (4,550-16,650)	5,490 (2,910-11,320) 68%	2,505 ( 790-5,330) 32%
0.5% Liver =275 $\gamma$ P. G. A.	36	$\frac{49}{48}$	+16.2 <sup>34</sup> (-6 to +33)	9,810 <sup>34</sup> (4,750-17,450)	6,895 (3,370-12,565) 70%	2,915 (1,115-5,815) 30%
1% Liver =550 $\gamma$ P. G. A.	35	$\frac{50}{49}$	+10.3 <sup>23</sup> (-23 to +35)	10,245 <sup>32</sup> (5,100-18,540)	7,555 (2,940-15,725) 72%	2,690 (1,020-6,305) 28%
Stock Diet I + lettuce	70	$\frac{50}{48}$	+19.6 <sup>64</sup> (-5 to +66)	10,850 <sup>43</sup> (6,550-17,300)	7,675 (3,700-14,530) 70%	3,175 (1,280-6,700) 30%

\* P. G. A. = Synthetic Pteroylglutamic Acid (Liver *L. casei* factor).

† Liver eluate powder (Lederle, Lab. No. 7-5293) supplying 550  $\gamma$  *L. casei* factor (pteroylglutamic acid)/g.

significantly improved at the two higher levels (275  $\gamma$  and 550  $\gamma$ /100 g. of diet), while the maximal effect on the body weight and circulating leucocytes of the lactating mother is shown at the lowest level (110  $\gamma$ ). Increasing the vitamin intake five-fold (550  $\gamma$  level) did not result in further improvement in these two criteria. There is very little variation in the weight change of the lactating mother from one series to another, regardless of the level given, *i.e.*, the averages for the different series are 8.6, 6.6, 5.5, 6.5, and 7.7 g., with an average value for all three levels of 7.0 g. The weaning weights of the young on the two higher levels likewise check very closely. The average leucocyte count (total) for different series varies from 7,000 to 8,000 with an average value

for all three levels (65 rats) of 7,490 cells. Pteroylglutamic acid has a greater effect on granular cells than on non-granular cells as can be seen by the return to the normal percentage of granulocytes (*i.e.*, 30%).

This improvement in lactation and in maternal leucocytes, although striking, is not sufficient to result in a normal picture (see stock Diet I, Table III). The weaning weights of the young and the weight gain of the mother on the supplemented diet are still below normal. The maternal leucocytes are normal only in the proportion of cell types; the number of circulating leucocytes does not attain the normal average and the ranges given for the total and differential counts show that some rats are still leukopenic. Almost 50% of the rats are below the 6,000 level for total leucocytes and 15% are below 1,250 granulocytes. However, only one rat in 65 (4%) dropped to the pathological level for granulocytes (<750 cells) in comparison to 60% on the unsupplemented purified diet. The data in Table III clearly show that while pteroylglutamic acid is one of the factors required during lactation on a purified diet, an additional unknown factor(s) is still necessary.

#### *Liver Eluate Powder*

Levels of the liver eluate powder (*L. casei* factor concentrate) corresponding to the previous levels of the synthetic pteroylglutamic acid were given, three series of litters being used for each level. The beneficial effects of the liver powder (Table III) are significantly greater than those for the corresponding levels of the synthetic vitamin and, moreover, show progressive improvement with each higher level. At the lowest level of liver powder (0.2%) there is a marked improvement in the weaning weights of the young. At the two higher levels (0.5% and 1.0%) the liver powder is significantly better than the synthetic vitamin by all criteria, *i.e.*, weaning weights of the young, weight change of the mother, and maternal leucocytes. There is little difference in the performance on the two higher levels of the liver powder. Mothers on the 0.5% level gained more weight during lactation while those on the 1% level weaned slightly heavier young and have a slightly greater number of total leucocytes, though not of granulocytes. There is much more variation in leucocytes for the 1% level from series to series than in the 0.5% level (it is possible that the slight diarrhea occurring in the 1% group decreased the beneficial effects of the liver powder).

At parturition the mothers receiving the liver-supplemented diet (all levels) averaged 170 days of age and 261 g. in body weight. They weaned 98% of their young and ate 32.9 g. of food daily. The intake of *L. casei* factor for the three levels was 36, 92, and 181  $\gamma$  daily, respectively. The corresponding values for the rats receiving synthetic pteroylglutamic acid were 179 days of age, 256 g. in body weight, 95% of young weaned, and 29.9 g. of food consumed daily. The vitamin intakes for the three levels were 30, 81, and 165  $\gamma$  daily, respectively. The difference in food intake correlates with the differences observed in lactation and maternal leucocytes and confirms the presence in liver powder of an unknown factor(s) essential for the rat during lactation.

### DISCUSSION

The production of a "folic acid" deficiency in the rat by means of sulfonamide-containing purified diets and its cure and prevention by low levels of the synthetic vitamin are well known (11). In rats maintained on purified diets without sulfonamides, Kornberg, Daft and Sebrell (12) found a small percentage (3%) with granulocytopenia which was corrected by crystalline *L. casei* factor. Likewise Skeggs and Wright (13) recently observed an incipient "folic acid" deficiency, as judged by leucocyte counts and hepatic storage of the vitamin, in rats maintained on purified diets for 9-10 weeks.<sup>7</sup> Toxic agents such as thiourea (15, 16) and promin or promizole (17) will produce blood dyscrasias in the rat for which "folic acid" concentrates or crystalline *L. casei* factor are beneficial. Furthermore, "stress" factors, such as hemorrhage (18), reduced caloric intake (19), pantothenic acid deficiency (20) and riboflavin deficiency (21), may be used to reveal latent "folic acid" deficiencies which would not otherwise be manifest. Lactation can now be added to the conditions ("stress" factors) by which this vitamin deficiency can be produced in the rat.

In the non-lactating rat maintained on purified diets with or without sulfonamides, curative doses of 20-25  $\gamma$  *L. casei* factor daily have been

<sup>7</sup> In this laboratory rats maintained on the basal purified diet 836 from weaning for several months have had normal blood counts. One group of nine older rats (220 days of age) averaged 9,995 total leucocytes of which 14%, or 1,440, were granulocytes. This may be compared with the data of Skeggs and Wright (13) in which rats, 84-90 days of age, averaged 7,950 leucocytes of which 8%, or 985, were granulocytes. The resistance of our rats to a "folic acid" deficiency after weaning is probably due to the use of 24% casein purified diets in place of the usual 18% level of casein. Wright and Skeggs (14) have emphasized the importance of the casein level in this respect.

used successfully (11, 12, 20, 21, 22). Daily prophylactic dosages of 2.5–4.0  $\gamma$  (which may or may not be optimal) have been reported (18, 23). In the lactating rat maintained on a purified diet in which the casein is probably not free of "folic acid", a daily prophylactic intake of 33  $\gamma$  (possibly less) is required for maximal effects on maternal body weight and circulating leucocytes and 33–81  $\gamma$  for the maximal effect on the weaning weights of the young.

The failure of these high levels to produce optimal effects on lactation and maternal leucocytes appears to be similar to the partial cures obtained by Daft *et al.* with high levels of the vitamin on the blood dyscrasias produced by thiourea + thyroxin (16) and to the blood dyscrasias in rats deprived of pantothenic acid (20) which do not always respond to the combined therapy of pantothenic acid and *L. casei* factor. Daft and Sebrell (23) have recently reported that pantothenic acid-deficient rats receiving *L. casei* factor prophylactically, respond better to whole liver powder than to pantothenic acid, thus indicating the presence of at least one unknown factor in the whole liver powder. Other investigators have reported the presence of unknown factors in liver for rats (24, 25, 26), for mice (27), for monkeys (28), for dogs (29), and for chicks (30, 31, 32). Some, but not all, of these factors have been differentiated from the different forms of "folic acid" (free and conjugated).

The addition of the liver eluate powder to the basal purified diet 836 resulted in a notable improvement in lactation and maternal leucocytes. Yet small differences between the normal (stock) values and the values obtained with the liver-supplemented diet (0.5% and 1% levels) occur consistently and may be significant. Further studies on these liver-supplemented diets are in progress.

#### ACKNOWLEDGMENT

The authors wish to acknowledge gratefully the able assistance of Freeke van Nouhuys and David H. Reeder in carrying out the hematological aspects of this problem.

#### SUMMARY

One hundred and forty litters have been used to establish standard values for lactation and maternal leucocytes for rats maintained on the stock diet and for those placed on the basal purified diet 836 at parturition.

The addition of high levels of synthetic pteroylglutamic acid (110  $\gamma$  level) results in striking improvement in maternal leucocytes and in the maternal body weight. Increasing the vitamin intake five-fold does not result in further improvement. A higher minimum level (275  $\gamma$ ) is necessary for significant improvement in the weaning weights of the young and doubling this intake is not more beneficial.

Levels of a liver eluate powder furnishing an equivalent amount of the *L. casei* factor, result in significantly greater improvement in lactation and maternal leucocytes than the synthetic vitamin.

The data given show that while pteroylglutamic acid is one of the factors required by the rat during lactation on a purified diet, an unknown factor(s), present in the liver eluate powder, is also necessary during this period.

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# Milk Studies. I. Some Vitamin and Trace Elements Found in the Colostrum of the Dairy Cow, Beef Cow and Swine<sup>1,2</sup>

R. W. Luecke, C. W. Duncan and R. E. Ely  
with the technical assistance of Elva Jones,  
Alice J. Greene and Carolyn Tull

*From the Departments of Agricultural Chemistry and Dairy, and the  
Institute of Nutrition, Michigan State College, East Lansing*

Received February 24, 1947

## INTRODUCTION

The vitamin and mineral content of colostrum milk is of the utmost importance for the newborn animal because it represents the sole source of nutrients for the first few days of life. The carotene and vitamin A content of the colostrum of the dairy cow has been investigated by Dann (1), Semb, Baumann and Steenbock (2), Stewart and McCallum (3), and for Shorthorn cattle by Henry, Houston and Kon (4).

Recently Pearson, Darnell and Weir (5) determined the B-vitamin content of the colostrum and milk of both the cow and ewe. They found not only a definite species difference but also higher concentrations of the B-vitamins in colostrum milk of the ewe than were found in that of the cow.

There are few data on the trace mineral composition of colostrum milk of the cow. Sato and Murata (6) found that cow's colostrum contained much more manganese than normal milk. Further studies on the mineral composition of cow's colostrum were carried on by Provan (7) and by Garrett and Overman (8). In general, it was found that the concentrations of calcium, phosphorus, magnesium, sodium and chlorine were higher in colostrum than in milk.

<sup>1</sup> Published with the approval of the Director of the Michigan Agricultural Experiment Station as Journal Article No. 860 (N.S.).

<sup>2</sup> This study was supported in part by a grant from the American Dairy Association.



In the present experiment it was desired to obtain the vitamin A, carotene and B-vitamin contents of colostrum milk of the dairy cow, beef cow and swine. Since the trace minerals play an important part in the nutrition of the bovine it seemed likewise advisable to determine the iron, copper and cobalt contents of colostrum milk. In the case of swine the small size of the sample prevented the determination of any of the minerals present.

From a review of the literature it is obvious that very limited studies have been made on the vitamin content of colostrum and that no specific information is available concerning the iron, copper and cobalt contents of bovine colostrum. The object of this paper is to report the concentration of the above constituents found in bovine and swine colostrum.

The colostrum samples taken from dairy and beef cattle represented 36-hour composite samples. The swine colostrum samples were taken within 3 hours following parturition.

The vitamin A and carotene were determined by a modification of the method of Boyer *et al.* (9) and thiamine by the fluorometric method of Hennessy (10). The microbiological methods employed for the determination of the B-vitamins were those of Snell and Strong (11) for riboflavin; Neal and Strong (12) for pantothenic acid; and Krehl, Strong and Elvehjem (13) for nicotinic acid. Copper and iron were determined by suitable modifications of the A.O.A.C. procedures (14). Cobalt was determined by a modification of the method of Butler and Allen (15).

## RESULTS

Table I shows the average and range of values found for iron, copper and cobalt in the colostrum milk of both the dairy and the beef cow. There seems to be no breed difference in the mineral content, although the iron content of Holstein colostrum seems to be somewhat higher. This difference, in view of the wide variations in the iron content, does not seem to be of any significance. In the published data on the iron content of milk a wide variation in concentration is noted. Johnston (16) has reported values ranging from 0.114 to 0.650 mg. of iron/kg. of milk. Table I indicates that the iron content of colostrum milk for both the dairy and the beef cow is higher than the reported values for milk (16).

Reported values in the literature on the copper content of cow's milk varied between 0.09 and 0.30 mg./l. according to Krenn (17) and Dills and Nelson (18). On this basis, the copper content of colostrum

TABLE I

*The Vitamin and Trace Elements in the Colostrum of the Dairy Cow, Beef Cow and Swine*

	Jersey	Holstein	Beef	Swine
No. Samples	14	7	6	6
Fe mg./kg.	1.47 <sup>1</sup> 0.70-3.60 <sup>2</sup>	2.00 0.81-3.10	1.80 1.30-2.50	—
Cu mg./kg.	0.48 0.20-0.71	0.56 0.10-0.90	0.40 0.30-0.60	—
Co mg./kg.	0.007 0.004-0.012	0.005 0.001-0.010	0.006 0.004-0.011	—
Carotene $\gamma$ /100 ml.	347 167-684	100 38-154	129 70-223	24 9-37
Vitamin A $\gamma$ /100 ml.	401 207-658	52 0-200	145 54-225	60 13-122
Thiamine $\gamma$ /ml.	1.22 0.90-2.70	0.91 0.60-1.24	0.85 0.64-1.00	0.86 0.52-1.01
Riboflavin $\gamma$ /ml.	5.74 3.20-8.01	6.00 2.87-9.00	4.98 3.22-6.75	4.00 1.65-6.25
Pantothenic Acid $\gamma$ /ml.	2.08 1.02-4.20	1.98 1.20-3.11	1.71 0.69-3.20	1.05 0.60-1.70
Nicotinic Acid $\gamma$ /ml.	0.87 0.61-1.70	0.77 0.61-0.97	0.57 0.43-0.61	1.43 1.14-1.85

<sup>1</sup> Mean

<sup>2</sup> Range

milk is significantly higher than milk (Table I). No data are available on the cobalt content of cow's milk. Spectrographic analysis could not detect cobalt in human milk (19). In the present experiment the cobalt of colostrum milk from dairy and beef cows varied between 0.001 and 0.012 mg./kg. of colostrum (average 0.005 mg.).

The carotene and vitamin A contents of the colostrum of the dairy cow, beef cow and swine are shown in Table I. As was expected, the carotene and vitamin A values obtained for the Jersey breed were higher than those for the Holstein. The concentrations of carotene and vitamin A in the beef cow colostrum were significantly higher than those in Holstein colostrum. The samples of colostrum obtained from the six beef cows represent three different breeds (Aberdeen Angus, Hereford and Shorthorn) with two animals from each breed. The two samples of colostrum from the Aberdeen Angus cows were higher in

carotene and vitamin A than the colostrum from either the Shorthorn or Hereford breeds.

The carotene content of swine colostrum averaged 24  $\gamma$ /100 ml. and the vitamin A content averaged 60  $\gamma$ /100 ml. (Table I). These pigs received no supplements of vitamin A but they were being fed a supposedly adequate ration which contained 10% alfalfa hay. Of the six colostrum samples obtained from swine three different breeds are represented (Chester White, Hampshire and Berkshire) with two animals from each breed. No breed difference in the carotene and vitamin A contents of the colostrum milks could be noted from the limited number of samples available. Benham (20) has reported the vitamin A content of swine colostrum to vary from 291 to 564  $\gamma$ /100 ml.

The B-vitamin content of the dairy cow, beef cow and swine colostrums are shown in Table I. There does not seem to be any significant difference between the Jersey and Holstein colostrum with respect to the B-vitamin content, except that the thiamine content of Jersey colostrum appears to be about 35% higher. The weighted averages obtained from this study for the thiamine content of both Jersey and Holstein colostrum are approximately 58% higher than that reported by Pearson *et al.* (5) for cow colostrum. The riboflavin content of beef cow colostrum averaged 4.98  $\gamma$  while that of the Jersey and Holstein averaged 5.74 and 6.00  $\gamma$ /ml. respectively. These values confirm those of Pearson *et al.* (5) who found that the average riboflavin content of 32 samples of cow colostrum was 6.10  $\gamma$ /ml. From the limited number of animals used in this work, the nicotinic acid content of the dairy cow colostrum appears to be about 50% higher than that of the beef cow, whereas the thiamine and pantothenic acid contents are approximately 30 and 20% higher, respectively.

Table I shows the average riboflavin content of swine colostrum to be 4.00  $\gamma$ /ml., whereas the beef and dairy cow colostrums are 25 and 46% higher, respectively. Both beef and dairy cow colostrums contain more pantothenic acid than swine colostrum (approximately 63 and 95%, respectively). There appears to be no difference in the thiamine content of either pig or beef cow colostrum but the colostrum of dairy cows contains about 30% more thiamine. On the other hand, the nicotinic acid content of swine colostrum is 150 and 68% higher, respectively, than that found in the colostrum of the beef cow or the dairy cow.

## DISCUSSION

The iron and copper contents of colostrum milk of the dairy cow are higher than the published values for these minerals in whole milk. Colostrum contains small but significant amounts of cobalt ranging from 0.001 to 0.012 mg./kg. No important difference was found between concentrations of these trace minerals in the colostrum of the dairy and the beef cow under the conditions of this experiment.

The very low concentrations of carotene and vitamin A in swine colostrum are difficult to explain, especially since two of the six sows had been kept on rye pasture for 30 days prior to farrowing. The concentrations of carotene and vitamin A in the colostrum of these two animals were no greater than that found in the animals which had been fed a normal ration containing 10% alfalfa hay.

Among the three breeds of beef cattle represented in the six samples of colostrum the concentrations of carotene and vitamin A were highest in the Aberdeen Angus breed. In general, the concentrations of carotene in the colostrum milk of the beef cow are similar to those found in the Holstein but the vitamin A content is about three times as high as that found in Holstein colostrum.

Pearson *et al.* (5) reported that the average riboflavin content of bovine colostrum was 6.10  $\gamma$ /ml. while that of the ewe was 20.08  $\gamma$ /ml. Although the newborn pig doubles its birth weight in a shorter time than does the newborn lamb (7 to 10 days), the concentration of thiamine, riboflavin, pantothenic acid and nicotinic acid are significantly lower in the colostrum milk of the sow. This apparent anomaly may be accounted for by the relatively smaller size of the newborn pig in relation to the larger newborn lamb.

## ACKNOWLEDGMENT

The authors are indebted to Professor G. A. Branaman and Professor W. N. McMillen of the Department of Animal Husbandry for supplying samples of colostrum from beef cows and swine.

## SUMMARY

No marked breed difference was found in the iron, copper and cobalt content of colostrum milk of the Jersey and Holstein dairy cow. The concentrations of these minerals in the colostrum of the beef cow were essentially the same as the values obtained for the dairy cow.

The carotene and vitamin A content of colostrum from the Jersey cow averaged 347 and 401  $\gamma$ /100 ml. while the corresponding values for Holstein colostrum were 100 and 52  $\gamma$ /100 ml. The concentration of these constituents in the colostrum of the beef cow were 129 and 145  $\gamma$ /100 ml., whereas, swine colostrum contained only 24 and 60  $\gamma$  of carotene and vitamin A/100 ml.

The colostrum of the sow is significantly lower in riboflavin and pantothenic acid, but markedly higher in nicotinic acid than either the dairy or the beef cow. The thiamine content of sow colostrum is similar to beef cow and Holstein colostrum but lower than Jersey colostrum.

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# The Amino Acid Composition of Secretin

Pehr Edman and Gunnar Ågren

*From the Chemical Department, Karolinska Institutet, Stockholm, and  
the Department of Medical Chemistry, University of Uppsala*

Received February 27, 1947

## INTRODUCTION

The amino acid composition of the secretin molecule was partly investigated by Ågren in 1934 and 1939 (1, 2). Since then the methods of amino acid analysis have been further developed and the problem was accordingly reinvestigated.

## EXPERIMENTAL

The analyses were performed on a preparation of secretin phosphate prepared from the crystallized picrolonate according to the directions given by Ågren (1). The preparation still had the same biological activity as when freshly prepared in 1938 (2) and was characterized by the following data:

Activity/mg. substance.....	250 units
Moisture.....	4.33%
Ash.....	6.67%
Nitrogen (Kjeldahl).....	12.3% (13.8%)
Phosphorus (Pregl).....	0.829% (0.932%)
Sulphur (Grote-Krekeler).....	0.74% (0.83%)

The values in brackets are calculated on a moisture- and ash-free basis.

The hydrolysis was performed in a boiling mixture consisting of 16% hydrochloric acid and 45% formic acid as recommended by Miller and du Vigneaud (3). The time of hydrolysis was 72 hours. An inconsiderable amount of insoluble humin was formed. The hydrolyzate was then freed from hydrochloric and formic acids by evaporating repeatedly to dryness after addition of an amount of sulphuric acid equivalent to the nitrogen.

The hydrolyzate was then resolved into basic, neutral and acidic amino acids by electrodialysis employing the device described by

Theorell and Åkesson (4). The following distribution of nitrogen calculated in percentage of total nitrogen was found:

Cathode fraction.....	35.9%
Middle fraction.....	40.9%
Anode fraction.....	12.9%
Ammonia.....	9.3%
Recovered nitrogen.....	99.0%

A qualitative analysis of the amino acids in each fraction was first made using the paper chromatography of Consden, Gordon and Martin (5). The solvents described by Edman (6) were used. The following amino acids were identified: *Cathode fraction* (pyridine-amyl alcohol): lysine, arginine, histidine. *Middle fraction* (pyridine-amyl alcohol/*isobutyric-isovaleric acid*): cystine, glycine, serine, alanine, proline, valine, methionine, leucine and (or) isoleucine, phenylalanine, tyrosine.<sup>1</sup>

*Anode fraction* (*isobutyric-isovaleric acid*): aspartic acid, glutamic acid.

Quantitative analyses were made on some of these amino acids. Glycine was estimated using a micro modification of the method of Alexander, Landwehr and Seligman (7). For the estimation of alanine a method worked out by Åqvist (8) was used. Tyrosine was determined by the method of Thomas (9) and histidine by the method of Jorpes (10). Serine was determined by a micro modification of the method of Boyd and Logan (11), cystine by the method of Block and Bolling (12) and methionine by the method of McCarthy and Sullivan (13) with some of the modifications described by Csonka and Denton (14).

The results, calculated in percentage of total nitrogen, are tabulated below:

Glycine-N.....	3.60%
Alanine-N.....	5.21%
Tyrosine-N.....	1.94%
Histidine-N.....	5.95%
Serine-N.....	1.84%
Methionine-N.....	2.10%
Cystine-N.....	0.73%

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<sup>1</sup> In addition there were two faint spots with small  $R_F$ -values in both solvents, which could not be identified with any known amino acids. Possibly it is a question of decomposition products formed during the hydrolysis.

## DISCUSSION

For a protein of such a low molecular weight as secretin, the figure 5000 was obtained by Svedberg (Hammarsten, Ågren, Hammarsten and Wilander (15)) using the ultracentrifuge, the molar ratios of the constituent amino acids can serve as a contributory criterion of homogeneity. If then the molar ratios for glycine, alanine, tyrosine, histidine, serine, and methionine are calculated with the value for tyrosine as a base they are found to be 1.86, 2.69, 1.00, 1.02, 0.95 and 1.08 respectively. It may be seen that all quotients closely approach an integer. Further, the minimal molecular weight calculated on the assumption of 1 mol. of tyrosine per mol. of secretin is 5,250, which is in good agreement with the experimentally determined molecular weight (15). The value of total sulphur and cystine, however, would seem to suggest that the minimal molecular weight is about twice as much. The sulphur value of the crystallized preparation obtained in 1933 is in good agreement with the present one. The question of the minimal molecular weight will be reinvestigated by means of the ultracentrifuge method which has been further developed since the last determination.

## ACKNOWLEDGMENT

This investigation was aided by grants from the Therese and Johan Anderssons Memorial Foundation.

## SUMMARY

The following amino acids have been demonstrated in the secretin molecule: Lysine, arginine, histidine, aspartic acid, glutamic acid, cystine, glycine, serine, alanine, proline, valine, methionine, phenylalanine, tyrosine, leucine and (or) isoleucine. Quantitative data for some of the amino acids are given.

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# The Nutrition of *Salmonella*

Joshua Lederberg<sup>1</sup>

*From the Department of Botany and Microbiology, Osborn Botanical  
Laboratory, Yale University, New Haven, Connecticut*

Received February 28, 1947

A number of strains of *Salmonella* were studied to determine their nutritional requirements in an attempt to find material for genetic analysis (Lederberg and Tatum, 1946). A summary of these findings will be given here.

The majority of strains are capable of growth on a simple synthetic medium (Gray and Tatum, 1944) containing glucose, salts and asparagine, as listed in Table I. All strains were first tested to determine whether they could develop optimally on this medium, by taking very small inocula from slant cultures of the various strains into 10 ml. The strains which failed to show rapid growth were tested further to determine the supplementation that was required, using previously described techniques (*cf.* Burkholder, 1943).

Individual strains of which the requirements have been determined are listed in Table II.

Hohn and Herrmann (1936) have attempted to classify the *Salmonella* group according to growth in a synthetic medium. The strains were designated as "ammonstark" and "ammonschwach," respectively. It is likely that the basis of this distinction is not the ability to assimilate ammonia, but the ability to synthesize specific growth factors such as those listed in Table II. The utility of such a classification has been questioned by Edwards and Bruner (1940) and Kauffmann (1941) who found considerable nutritional variation among strains of similar serological and epidemiological behavior.

From the work of Gray and Tatum (1944) and Roepke *et al.* (1944) on *Escherichia coli*, and of Beadle and Tatum (1941) on *Neurospora*, it is likely that growth factor deficiencies of bacteria are the result of mutations of genes controlling specific steps in

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<sup>1</sup> Fellow of the Jane Coffin Childs Fund for Medical Research. This work was supported by a grant from the Jane Coffin Childs Fund and will be submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Yale University.

biochemical syntheses. For example, the ability of ornithine and citrulline to replace arginine in the growth of *S. enteritidis* (S45) suggests that these compounds are biosynthetic precursors of arginine, and that the formation of ornithine is prevented in this organism by the mutation of a gene controlling its synthesis (Srb and Horowitz, 1944).

TABLE I  
*Salmonella Types With No Growth Factor Deficiencies*

Type	Number of strains tested	Type	Number of strains tested
*S. paratyphi A	5	S. montevideo	6
* typhi-murium*	19	tennessee	1
san diego	1	georgia	1
reading	1	newport	11
derby	5	oregon	1
essen	1	glostrup	1
budapest	1	düsseldorf	1
california	1	amherstiana	1
brandenburg	1	* enteritidis	9
abortus equi	1	dar-es-salaam	1
abortus bovis	2	panama	3
breddeney	1	javana	1
* cholerae suis	4	london	1
thompson	1	give	1
oranienburg	4	uganda	1
bareilly	4	anatis	2
hartford	1	münster	1
zanzibar	1	onderstepoort	1
meleagridis	1	hvittingfoss	1
infantis	1	gaminara	1
pueris	1	kirkee	1
newington	1	kentucky	1
new-brunswick	1	minnesota	1
illinois	1	tel-aviv	1
senftenberg	1	ballerup	1
aberdeen	1	urbana	1
rubislaw	1	inverness	1
poona	1	adelaide	1
carrau	1	italiana	1
sendai	1 (slow on minimal)	champaign	1

Types marked \* also have representatives with growth factor requirements. Cf. Table II.

TABLE II  
*Deficient Strains of Salmonella*

Strain	Requirements
*S1 paratyphi A	methionine, tryptophan (or indole)
*S42 paratyphi A	tryptophan (or indole)
S51 paratyphi B	proline
*S4 cholerae suis	methionine
*S55 cholerae suis	tryptophan (or indole), cystine
*S56 cholerae suis	biotin, tyrosine
S36 gallinarum	thiamine
S37 dublin	thiamine
*S45 enteritidis	arginine (replaceable by ornithine or citrulline)
S12 pullorum	leucine, cystine
S14 pullorum	leucine, cystine, methionine
*S61 typhi-murium, IV-variant	methionine
S. paratyphi A	not determined
S. paratyphi B	not determined
S. typhi suis	not determined
S. abortus ovis	not determined

Types marked \* also have representatives without growth factor requirements.  
 Cf. Table I.

The persistence in the natural populations of these organisms of growth factor requirements, of mutational origin, can hardly be regarded as an accident. In the laboratory the loss of these requirements, presumably by reverse-mutation, is a relatively frequent occurrence both in *Salmonella* strains and in nutritional mutants of *E. coli* (Ryan and Lederberg). By plating large numbers of cells into agar lacking the specific growth factors, one may select for "reverted" types of *S. enteritidis* (S45) which are capable of synthesizing ornithine, of *S. cholerae suis* (S55) capable of synthesizing cystine, and so forth. To account for the maintenance of these requirements, it seems necessary to assume that the deficient types found in nature have a selective advantage over reverted types. An illustration of this sort of competition in laboratory populations has been found in *Neurospora* (Ryan and Lederberg, 1946).

It would be dangerous to draw conclusions concerning the evolutionary development of the *Salmonella* group from their nutritional behavior because of the demonstrated lability of these characters. The reversibility of requirements indicates, however, that the ability to synthesize growth factors is the more primitive stage (cf. Lwoff,

1942). It is unlikely that the function of synthesizing a required metabolite, could be so readily evolved, *de novo*. One may conclude, therefore, that nutritional reversion represents the reacquisition of a function, the gene for which had been only partially impaired. The common occurrence of reversible requirements suggests that there is also other latent genic material which, although propagated from generation to generation, has no demonstrable function. Such genes are perhaps available for the divergent mutations which are required for evolutionary progress.

#### ACKNOWLEDGMENT

The majority of the strains used in this investigation were kindly made available to us by Dr. K. M. Wheeler and Dr. E. K. Borman of the Connecticut State Public Health Laboratories, for which we express our grateful acknowledgment. We also wish to thank Dr. P. R. Edwards, Dr. L. Rettger, Dr. Erich Seligmann, and Dr. F. Kauffmann, for their courtesy in providing such cultures. The author takes pleasure in recording his appreciation to Professor E. L. Tatum for his encouragement and support.

#### SUMMARY

The nutrition of a variety of *Salmonella* cultures has been studied. The majority have no growth factor requirements; other strains requiring various amino acids and vitamins are described.

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# Trypsin Inhibitor. IV. Occurrence in Seeds of the *Leguminosae* and Other Seeds<sup>1</sup>

Raymond Borchers and C. W. Ackerson

with the technical assistance of

Leo Kimmett

*From the Department of Agricultural Chemistry, Nebraska Agricultural  
Experiment Station, Lincoln 1, Nebraska*

Received March 14, 1937

## INTRODUCTION

A trypsin inhibitor has been reported to be present in unheated soybean meal by Ham and Sandstedt (1) and in navy beans by Bowman (2) using *in vitro* methods. Kunitz (3) has crystallized a trypsin inhibitor from soybeans while Bowman (4) has recently presented evidence indicating the presence of at least two distinct trypsin inhibitors in soybeans. The soybean fraction containing the trypsin inhibitor has been shown to have growth-inhibiting properties for the chick by Ham *et al.* (5) and for the rat by Klose *et al.* (6). The importance of these findings prompted an investigation of other legumes for the possible presence of a trypsin inhibitor.

## METHOD

The seed <sup>2</sup> was ground and extracted with cold ether. The resulting meal was extracted in the proportion of 1 g. meal + 10 ml. extractant at 4°C. overnight. Extractants used were 0.05 *N* and 0.5 *N* hydrochloric acid and distilled water. The extract was clarified by centrifugation and trypsin inhibitor determined according to the method <sup>3</sup> of

<sup>1</sup> Published with the approval of the Director as Paper No. 414, Journal Series, Nebraska Agricultural Experiment Station.

<sup>2</sup> We are indebted to numerous Agricultural Experiment Stations and seed companies for graciously supplying seeds used in this study and to Dr. T.-H. Goodding, Department of Agronomy, University of Nebraska, for his kind advice.

<sup>3</sup> A 4% suspension of Pfanstiehl (1-110) trypsin powder clarified by filtration was used as the stock trypsin solution.

TABLE I

*Trypsin Inhibitor in Seeds of the Leguminosae and Others*

Description	Inhibitor Units/ml. Extract (equivalent to 0.1 g. defatted meal) $\times 10^{-3}$
Legumes <sup>1</sup>	
<i>Arachis hypogaea</i> —peanut	16.6
<i>Canavalia ensiformis</i> —jack bean	0
<i>Caragana arborescens</i> —Siberian pea tree	0
<i>Cassia tora</i> —sickle senna	0
<i>Ceratonia siliqua</i> —carob bean	41.4
<i>Cercis canadensis</i> —redbud tree	11.2
<i>Chamaecrista fasciculata</i> —partridge pea	6.5
<i>Cicer arietinum</i> —chick pea, garbanzo	32.3
<i>Cyamopsis psoraloides</i> —guar bean	0
<i>Gleditsia triacanthos</i> —honey locust	26.2
<i>Gymnocladus dioica</i> —Kentucky coffee bean	24.4
<i>Lens esculenta</i> —common lentil	0
<i>Lespedeza stipulacea</i> —Korean lespedeza	6.9
<i>Lupinus angustifolius</i> —blue lupine	0
<i>Medicago sativa</i> —alfalfa	8.6
<i>Melilotus alba</i> —white sweetclover	0
<i>Melilotus officinalis</i> —yellow sweetclover	0
<i>Mucuna deeringianum</i> —Florida velvet bean	11.5
<i>Phaseolus aureus</i> —golden mung bean	6.2
<i>Phaseolus coccineus</i> —scarlet runner bean	21.8
<i>Phaseolus lunatus</i> —lima bean	29.1
<i>Phaseolus vulgaris</i> —garden bean	44.1
<i>Pisum sativum</i> —garden pea	0
<i>Pisum sativum</i> var. <i>arvense</i> —field pea	0
<i>Soja max</i> —soybean	35.0
<i>Sophora japonica</i> —Japanese pagoda tree	19.7
<i>Trifolium pratense</i> —mammoth red clover	0
<i>Vicia faba</i> —horse bean	0
<i>Vicia sativa</i> —common vetch	0
<i>Vigna sinensis</i> —black eyed pea	43.7
Non-legumes	
<i>Aleurites fordii</i> —tung bean	0
<i>Avena sativa</i> —oats	0
<i>Hordeum vulgare</i> —barley	0
<i>Linum usitatissimum</i> —flax	0
<i>Secale cereale</i> —rye	0
<i>Sorghum vulgare</i> —Leoti	0
<i>Triticum vulgare</i> —wheat	0
<i>Zea mays indentata</i> —corn	0

<sup>1</sup> Nomenclature according to GRAHAM, E. H., Legumes for Erosion Control and Wildlife, U.S. Dept. Agr. Misc. Publ. No. 412 (1941).

Borchers *et al.* (7). The results appear in the accompanying table. The nutritional significance of these findings will be the subject of a later report.

### SUMMARY

The trypsin inhibitor found in soybeans is not characteristic of the seeds of all *Leguminosae*. Among the legume seeds of nutritional importance investigated, it was found in the peanut, chick pea, soybean, mung bean, scarlet runner bean, lima bean, garden bean, velvet bean, and black eye pea, but not in the jack bean, guar, lentil, garden pea and horse bean. The trypsin inhibitor was not found in the non-legumes investigated.

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# Effect of Quantities of Carotene in the Ration on the Fertility of White Rats and the Quality of the Young

G. S. Fraps

*From the Division of Chemistry (Texas Agricultural Experiment Station),  
A. & M. College of Texas, College Station*

Received February 14, 1947

## INTRODUCTION

H. C. Sherman and associates (7,8,9,10) have shown that the vitamin A value of the ration affects the length of life of white rats and the length of the reproductive period of the females. Hou (5), Newton (6), Browman (1), Evans (2), Tansley (11), and others have studied the effect of vitamin A on reproduction. The object of the work here presented was to ascertain the effect of various quantities of  $\beta$ -carotene upon reproduction of white rats. In vitamin assays by the modified U. S. P. method used in this laboratory (3), the young rats at the age of 21-28 days should weigh 40-50 g., should become depleted on the vitamin A-free ration in 45 days or less, and weigh 70-130 g.

## EXPERIMENTAL

At the time of weaning, groups of 10 females and 4 males were placed upon diets containing 0.5, 1.0, 2.0, 4.0 and 10.0 parts per million  $\beta$ -carotene in the form of alfalfa leaf meal. The basal ration consisted of corn meal 1200 g., cottonseed meal 300, powdered skim milk 500, Wesson oil 100, yeast 100, irradiated yeast 10, and salt mixture 21.5 g. The alfalfa leaf meal was analyzed (4) from time to time, and added to the basal ration in the quantity needed to supply the carotene specified. The females were mated at the usual breeding age with males fed the same level of carotene. The young rats produced were placed on the vitamin A-deficient diet to ascertain the time of their depletion in vitamin A.

## RESULTS

The results are given in Table I. With increasing quantities of carotene for 0.5 to 8.0 parts per million, there were increases in the number

TABLE I  
*Effect of Quantities of Carotene on Production and Quality of Young Rats*

	Carotene 0.5 p.p.m.	Carotene 1.0 p.p.m.	Carotene 2.0 p.p.m.	Carotene 4.0 p.p.m.	Carotene 8.0 p.p.m.
Daily intake of carotene, $\gamma$ , per female	3.6	6.8	22.4	38.2	86.1
Number of litters	24	27	31	33	36
Total number of young	154	212	216	244	251
Percentage born alive	68	80	90	90	96
Percentage weaned of those born alive	52	69	80	63	81
Percentage weighing less than 40 g. when weaned	67	28	10	0	0
Percentage weighing 40-50 g. when weaned	33	72	90	96	94
Percentage weighing more than 50 g. when weaned	0	0	0	4	6
Percentage of those weaned suitable to place on test diets after depletion	23	60	81	90	87

of litters, the number born, and the percentages born alive. The percentage weaned of those born alive increased up to and including 2 p.p.m. of carotene. The low percentage weaned in the group receiving 4.0 p.p.m. was due to cannibalism of the mothers. The percentage weighing less than 40 g. when weaned decreased from 67% with 0.5 p.p.m. carotene to zero with 2 p.p.m. carotene. Those weighing 40-50 g. when weaned increased from 33% with 0.5 p.p.m. carotene to 96% for those receiving 4 p.p.m. carotene. After depletion on a vitamin A-free diet, the rats weaned suitable for use increased from 23% on 0.5 p.p.m. of carotene to 90% on 4 p.p.m. of carotene.

The level of 4 p.p.m. carotene was considered to be best suitable for the production of rats for vitamin A work on the ration used.

Grateful acknowledgment is made for the assistance of W. W. Meinke.

#### SUMMARY

Rats were fed on a basal ration supplemented by 0.5, 1.0, 2.0, 4.0 and 8.0 parts per million of  $\beta$ -carotene. As the amounts of carotene increased, there were increases in the number of litters, the number of young, the percentage born alive, and the live weight of the young rats at time of weaning. The percentage weaned of those born alive

reached the maximum of 80 at 2 parts per million of  $\beta$ -carotene. After depletion of vitamin A, those suitable for use in the biological method to determine vitamin A reached the maximum of 90% of those weaned on 4 p.p.m. of carotene.

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## LETTERS TO THE EDITORS

### Vitamin E in Laboratory Diets

While it is obvious that the place of the tocopherols in practical nutrition cannot be properly ascertained by comparing the performance of laboratory animals on fortified diets with those on diets scrupulously free of vitamin E, it has not become generally recognized that laboratory animal diets which contain several *per cent* fresh or hydrogenated vegetable oil already contain more tocopherols than most human diets or the diets of many farm animals. Addition of more vitamin E to such an experimental diet is, for most purposes, superfluous.

Some reports of carefully performed researches in this field are unconsciously but thoroughly misleading. A recent example (1) reports that  $\alpha$ -tocopherol did *not* effect the utilization of carotene in spinach; but only after a careful search of sections printed in fine type would it be clear to the reader that both the test and the control diets already contained enough vitamin E to afford optimum utilization of the carotene. No significant improvement could be expected from further additions of tocopherols.

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Research Laboratories,  
Distillation Products, Inc.,  
Rochester 13, N. Y.  
February 26, 1947.

NORRIS EMBREE

### Relation Between pH and the Effect of Pyruvate on the Desamidation of Glutamine and Asparagine

Sirs:

At pH 6.7-6.8, glutamine and asparagine are relatively slowly desamidated by aqueous extracts of rat liver; when pyruvate is added to the

digest, the rate of desamidation of these amino acid amides is considerably increased (*1*). The effect of the pyruvate is optimal at a ratio of 2 moles of the keto acid to 1 mole of amide. The pyruvate is not consumed by the reaction, and the ammonia which appears in the digest is quantitatively accounted for by the decrease in amide group nitrogen.

Fig. 1 describes the effect of pH on the desamidation of glutamine and asparagine by rat liver extracts in the presence and absence of

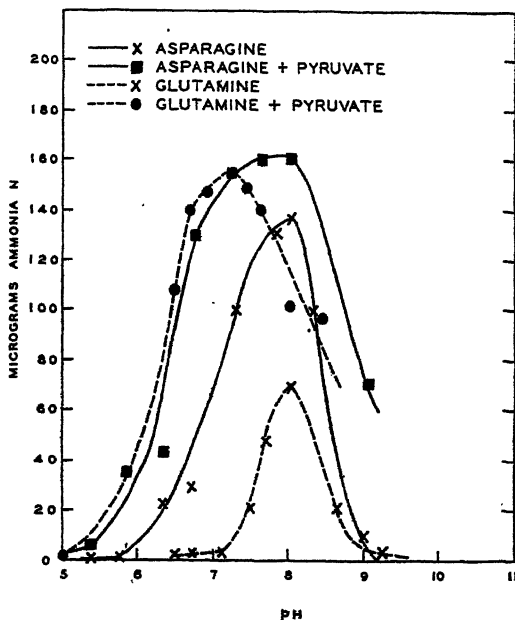


FIG. 1. Effect of pH in the desamidation of glutamine and asparagine by rat liver extracts in the presence and absence of added pyruvate.

added pyruvate. The digests consisted of 1 cc. of fresh, aqueous extract of rat liver equivalent to 333 mg. tissue, 1 cc. of veronal buffer, 1 cc. of glutamine or asparagine at 0.014 *M* concentration, and 1 cc. of sodium pyruvate at 0.025 *M* concentration. The incubation period was 4 hours at 37°C., and the results are given in terms of  $\gamma$  of ammonia N which appear in the digests corrected for the extract blanks. The optimum for glutaminase and asparaginase activity is about pH 8; in the case of the pyruvate effect on the desamidation of glutamine, the optimum is

shifted to about pH 7. Due to the high asparaginase activity of liver, the effect of pyruvate on the desamidation of asparagine is appreciable only at about 6.7–6.8. Between pH 6 and pH 7.2 where glutaminase activity is minimal, the desamidation of glutamine in the presence of pyruvate is considerable.

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*National Cancer Institute,  
National Institute of Health,  
Bethesda, Maryland,  
March 17, 1947.*

JOSE M. GONÇALVES <sup>1</sup>  
MAURICE ERRERA <sup>2</sup>  
JESSE P. GREENSTEIN

### Effect of Heating on the Capacity of Rat Liver Extracts to Desamidate Glutamine and Asparagine

Sirs:

The desamidation of glutamine and of asparagine by rat liver extracts is considerably increased in the presence of added pyruvate (1). In the hope of separating the effect of pyruvate on the desamidation of these amides from glutaminase or asparaginase activity itself, the extracts were subjected to various heating procedures prior to the digestion experiments.

Fresh aqueous extracts of rat liver, equivalent to 333 mg. tissue/cc. and at pH 6.8, were heated at various temperatures for 10 minutes, quickly chilled, brought to room temperature, and 1 cc. aliquots mixed with 1 cc. veronal buffer to bring the pH to 8.0, 1 cc. of glutamine or asparagine at 0.014 *M* concentration, and 1 cc. of sodium pyruvate at 0.025 *M* concentration. The incubation period was 4 hours at 37°C., and the results are given in terms of  $\gamma$  of ammonia N which appear in the digests corrected for the extract blanks. The data reveal that prior heating of the extract for 10 minutes at 50°C. results in a nearly complete loss of glutaminase and of asparaginase activity, but in very little loss in the desamidation of the amides in the presence of pyruvate. These results have been repeatedly confirmed, and form a basis for present studies on the isolation of the systems responsible for the desamidation of the amides in the presence of pyruvate. It appears

<sup>1</sup> Rockefeller Foundation Fellow, on leave from University of Brazil.

<sup>2</sup> On leave from the University of Brussels.



unlikely that the effect of pyruvate is simply an augmentation of glutaminase or asparaginase activity, but rather that the pyruvate forms some intermediate with the amide, perhaps of the nature of a dehydropeptide, which is then hydrolyzed at a relatively high rate to yield ammonia and the regenerated pyruvate (1).

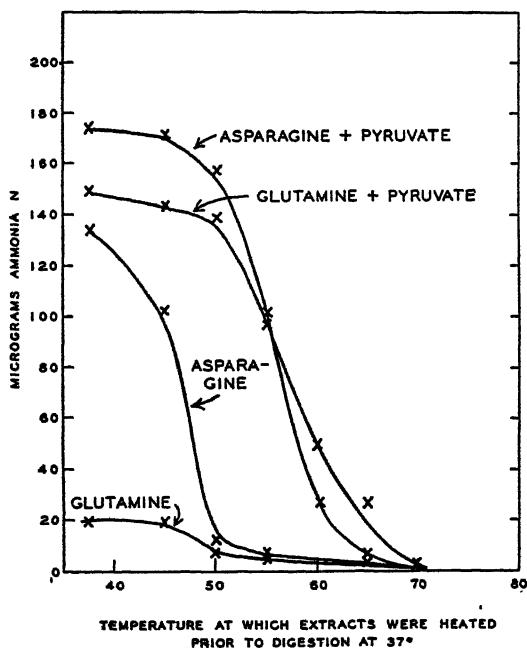


Fig. 1. Amide nitrogen as ammonia split at pH 8 from digests of glutamine and asparagine alone and in the presence of added pyruvate with aqueous rat liver extracts which had been heated for 10 minutes at various temperatures prior to digestion of the substrates at 37°C.

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National Cancer Institute,  
National Institute of Health,  
Bethesda, Maryland.

March 17, 1947.

MAURICE ERRERA<sup>1</sup>  
JESSE P. GREENSTEIN

<sup>1</sup> On leave from the University of Brussels.

## Book Reviews

**Manuel de biochimie.** By PIERRE THOMAS de l'Institut Pasteur, chargé de cours à la faculté de médecine de Lausanne. Deuxième édition remaniée. Masson et Cie, éditeurs, Paris, France, 1946. x + 999 pp. Pricé 1500 Fr. = \$15.00.

Despite the international character of science the specific talents of a people find their expression, particularly in textbooks, in the different presentation of the subject matter. This applies both to the mastery and grouping of the material and to the consideration given to the foreign literature.

In this respect the "Manuel de biochimie" by Pierre Thomas is quite unfrench. One notices immediately that the author is completely familiar with the world literature of his field. What has been done in other countries is fully acknowledged. None of the important results achieved during the last 50 years is missing. Pierre Thomas' manual differs remarkably from many a modern compendium in the loving care and attention given to the fundamentals of research. He did not discard what is omitted in many modern textbooks, usually due to ignorance of the authors, namely, the development of our present knowledge. Especially in biochemistry the best representatives of the field always kept to the preparative side, while many lesser men brought forth a mass of publications often of doubtful or limited value, by applying in a comfortable routine manner indirect methods which generally are of real value only in the hands of their creators and masters. Thomas correctly evaluates the preparative side.

Chapters on the physicochemical aspects of the cell and organism (atom, molecule, gases, liquids and solids), the colloidal state, colloidal macromolecules, adsorption, chromatography, viscosity, osmotic pressure, catalysis in general and specifically enzymes, form the introduction of the manual. The second part is concerned with proteins and their degradation products, nucleo- and glycoproteins, lipoids and fats, sugars and polysaccharides, and the mineral content of the cell. The third part bears the characteristic title "Synthesis, dislocation and desmolysis," Starting with photosynthesis, the formation of lipoids, purines, proteins, pyrimidines, the physiological and pathological degradation of all these substances, the desmolysis and intermediary steps of desmolysis, conjugation with  $H_2SO_4$  and glucuronic acid, formation of hippuric acid, acetylation and methylation are discussed. The fourth part consists of a description of tissues and their functions, blood, blood pigment, plasma and serum, gas and alkali reserve, lymphs and transudates, bone marrow, vitamins, hormones, the digestive system, liver, bile, muscle and muscular exertion, nerves and sensory organs, production of egg, milk, kidney secretion, perspiration and feces.

All this subject matter has not been treated in a dry way or just enumerated within the respective chapters but connected organically and well organized. To each important chapter Thomas further appended directions for simple and yet cleverly chosen practical experiments, thus giving the book a special charm and didactic

value which saves both student and teacher from the worst of all methods—the lecture without experiments.

If one further considers that the text is supplemented and extended by carefully arranged tables and reliable data as well as lucid descriptions of methods and instructive illustrations, Thomas' book will be recognized as a true "manual". Referring to monographs, collective volumes and compendia the author was able to avoid too involved a bibliography. A special appeal lies in the last 100 pages, treating the biochemistry of plants; this field, which has always been specially cherished in France, is brought out in an effective and instructive manner. The author, himself active in many branches of biochemistry, presents his readers with a book which is both useful and pleasing.

CARL NEUBERG, New York

**Pflanzliche Infektionslehre.** By ERNST GÄUMANN, O. Professor für Spezielle Botanik an der Eidg. Techn. Hochschule in Zürich. Verlagbirkhäuser, Basel, Switzerland, 1946. 611 pp. Price, Swiss Fr. 48.50

"Pflanzliche Infektionslehre" is the best modern compendium of information regarding the factors affecting the development of plant diseases caused by plant pathogens. The subtitle "Lehrbuch der allgemeinen Pflanzenpathologie für Biologen, Landwirte, Förster und Pflanzenzüchter" implies that the book is intended as a text. Even if never widely used as a text, it should be. The basic plan of the book is sound and is developed logically and with scholarly discrimination and accuracy. The principles are illustrated by well-chosen examples, without a plethora of detail that might confuse rather than clarify. The book will appeal especially to those students who are not satisfied to know merely what happens but want to learn also why it happens, insofar as possible. This is no handbook or elementary text; it is a scholarly treatise on the principles underlying the development of plant diseases. For this reason it will be welcomed by plant pathologists and other plant scientists who are interested in principles rather than mere descriptions of diseases and prescriptions for their control.

The book is divided into six chapters, each one of which is subdivided in such a way as to make the Table of Contents a detailed guide to the materials in the book. Chapter 1 considers the phenomena of infection, including the modes of entrance of various kinds of pathogens into host plants under different environmental conditions, the time required for infection, incubation and fructification of the pathogens, the avenues of entrance, and the establishment of the pathogen in the host. In Chapter 2 are considered the sources of inoculum, methods of dissemination, and the development of epidemics. This is followed in Chapter 3 by a discussion of the pathogenic potentialities of pathogens, including genetic differences and variability due to nuclear phases in life cycles, genetic and physiologic changes in the pathogens, host influences, and the mutual effects of organisms in mixed infections. Chapter 4 deals with the nature and variability of disease resistance and susceptibility. It includes a detailed discussion of developmental, morphologic, and physiologic types of resistance, including immunization and inheritance of disease resistance. This is followed by a detailed discussion of factors that predispose host plants to disease. Chapter 5 is devoted to the nature of diseases, including general signs and symptoms, and morphological and physiological changes in diseased plants. Finally, in Chapter 6 are given the gen-

eral principles on which control measures are based: prevention of infection; the use of resistant varieties and maintenance of resistance by means of proper soil management; and what is known about chemotherapy.

The sequence of treatment, then, is as follows: How does the pathogen enter the host; how does it get to the host; what are the prerequisites for the development of disease, both as concerns the pathogen and the host; what are the manifestations of disease; and how can diseases be prevented? Whether this sequence is best is a matter of personal preference. For many years I have used one that differs somewhat from that of Professor Gäumann, attempting especially to summarize pertinent information about the nature, multiplication, liberation, and dissemination of pathogens before considering avenues and modes of entrance. In my plan the development of epidemics also has been considered later in the sequence of events than in Professor Gäumann's, as it is based on many of the facts that he gives after a discussion of epidemics. There are advantages and disadvantages in both systems.

There is commendable unity and coherence in Gäumann's exposition. This is, of course, vindication of the wisdom of grouping facts around phenomena. It is a particularly noteworthy achievement when one remembers that there are thousands of varieties of hundreds of species of plants that may be parasitized by innumerable parasitic races of numerous species of pathogens, belonging to many taxonomic categories. From the multiplicity of data, Gäumann has chosen wisely and well.

That the emphasis in the book is on basic principles rather than practical applications is clear from the fact that the first three chapters are of almost equal length and comprise about one-third of the 569 pages of text; chapter 4 has more than 200 pages; chapter 5, about 50 pages; and chapter 6, on control, is only a little more than 5 pages long. Moreover, the examples that illustrate principles are selected because of their pertinence rather than because of economic importance. And yet the book is useful from a practical standpoint because it summarizes the most important known facts in connection with disease phenomena. As an example, a plant breeder can find good summaries of the effects of such factors as nutrition, temperature, humidity, light, and soil conditions on the susceptibility of plants to disease. Likewise, pathologists can easily obtain the most important information regarding such subjects as variability of pathogens, pathological histology, and metabolic disturbances caused by diseases.

The book is thoroughly modern in facts and viewpoints. Pertinent facts regarding the physiology, ecology, and genetics of plant pathogens are used to explain and interpret pathogenicity and its variability. Similarly, facts regarding the morphology, physiology, ecology, and genetics of host plants are used to explain the reactions to the entrance and development of the pathogen.

Illustrations, graphs, and tables are used freely, and add much to the ease of comprehension. The book is well written, and the approximately 700 references to literature are useful guides to those who wish to consult original sources of information.

"Pflanzliche Infektionslehre" is witness to the fact that plant pathology is a pure science as well as an applied one. It is evidence also that it is a very complex science, requiring knowledge and techniques of many of the basic sciences, such as physics, chemistry, the various branches of botany, soils, and genetics, with mathematics being used increasingly as the language of the science. But, above all, the author deserves

high commendation for his continued successful efforts to concentrate on basic principles and thus help promote progress in both the pure and applied phases of plant pathology.

E. C. STAKMAN, St. Paul, Minn.

**Organic Chemistry.** By PAUL KARRER, Professor at the University of Zurich, Switzerland. Translated by A. J. MEE, Head of the Science Department, Glasgow Academy. Second English Edition, revised and enlarged. Published and distributed in the public interest by authority of the Alien Property Custodian. Elsevier Publishing Company, Inc., New York, N. Y., 1946. xx + 953 pp. Price \$7.50.

Karrer's well known textbook has enjoyed increasing popularity, especially in European countries, since the first German edition was published in 1927. It doubtlessly also influenced a number of teachers who have conducted classes in Organic Chemistry. The size of the book, nearly a thousand pages, differentiates it from smaller and, in some cases, schematically presented undergraduate texts on one hand, and from the monumental but unfinished German textbook written by V. Meyer and P. Jacobson on the other.

The reviewer believes that in addition to the well chosen scope and very clear grouping of the material, the following three features make this textbook especially valuable for the student.

1. The respective sections of systematic organic chemistry are well balanced, as shown by the following space data: aliphatics, 332 pages; aromatics, 264 pages; alicyclics, 108 pages; and heterocyclics, 150 pages.

2. The book reflects a main trend of the twentieth century organic chemistry, namely, the steadily increasing interest in the study of natural products, especially of those whose importance has been recognized in bioassays.

3. The book under review contains thousands of structural formulae which are clearly expressed in every detail. The replacement of a benzene ring by  $\phi$  or of a long side chain by  $R$  may be agreeable for the printer, but it certainly does not give the reader a precise notion of the shape of the molecule. Since most students belong to the visual type, the neglect of complete formulation seems to work against their acquiring familiarity with the language of organic structures.

Some conversions can be found which are more or less familiar to the average American chemist but do not occur in Karrer's book. However, one could also find examples which point in the opposite direction. Since, in spite of the internationality of science, the main lines of research and teaching also follow some traditions in the respective countries, it seems that a good textbook originating from abroad may be of stimulating effect to many students and a welcome addition to their libraries.

The foregoing remarks may show the importance of translating Karrer's textbook. The following critical comments may assist in making the influence of its English version as effective as possible.

The original of the work under review is a "Lehrbuch" and the word "Textbook" should have been kept in the title. Certainly, the large volume constitutes also a very handy reference book. However, this is by no means its essential feature. Those paragraphs which are intended mainly for reference are printed in small type, and it would perhaps, even be desirable to increase moderately the fraction thus printed.

It is regrettable that the present translation is based on the eighth German edition which appeared in Leipzig in 1942 and, evidently, reflects the state of our knowledge as of 1941. Although it is indicated on the reverse of the title page that some additions and revisions have been made, these can be qualified only as a retouch.

One may expect from a good textbook that it should refer to those literary sources which can guide the student farther. A stimulus in this direction was given by the author by printing the titles of chosen monographs in footnotes at the beginning of each main chapter. Unfortunately, these references have not been critically revised for the present English edition. Indeed, a number of the books quoted are so antiquated that they may even mislead the student as to the present state of the literature. Just to give a few examples: On page 467, Schwalbe's "Newer Theories of Dyeing" is quoted (40 years old); p. 733, Wedekind's "Heterocyclic Compounds in Organic Chemistry" (46 years old); p. 347, Schwalbe's "Chemistry of Cellulose" (36 years old); and on p. 575, Gnehm's "Anthracene Dyes" (50 years old). The translator could also have included advantageously the titles of good monographs which were published during recent years in the United States or England.

A modernization of the analytical introduction would also be desirable. Furthermore, the importance of methods like chromatography or spectroscopy should be emphasized and illustrated by examples.

It has been suggested that the book should have gone deeper into the application of modern concepts based on electronic structures. However, the reviewer believes that this would require a further very substantial increase of the volume. It seems that at the present time the treatment as given by Karrer cannot be replaced by the theories indicated in a truly general manner.

Binding and printing are remarkably good and the price of the book is reasonable. A multitude of symbols is clearly arranged and faultlessly reproduced. In a very few formulas, *e.g.*, in that of cozymase (p. 714), some valency lines are missing. The photographs facing page 8 are inferior to those of the German original (5th edition). The excellent Index covers 28 pages and is complete as to names of compounds. However, the student would like to find in this index also some more general terms like methylation, alkylation, decarboxylation, ring closure, hydrogen addition, double bond migration, *etc.*, as well as such synonymous names as are preferred in the Anglo-Saxon countries but which do not figure in the present index or text (epinephrine = adrenaline, cincophene = atophane, *etc.*).

Summarizing, it can be stated that the second English version of Karrer's textbook is a valuable addition to the list of those publications which are expected to be used extensively in all English-speaking countries. It would be well worth the effort to keep it on the market and up to date for a long time to come.

L. ZECHMEISTER, Pasadena, Calif.

**Nomograms for Manometer Constants.** By MALCOLM DIXON, Cambridge University. Cambridge, at the University Press; New York, The Macmillan Company, 1946. 6 pp. Price \$0.25.

This short booklet contains nomograms which permit one to determine rapidly the constants ( $k_{CO_2}$ ,  $k_{O_2}$ ) at 25°C. and 37°C. of the Warburg (constant volume) and the Barcroft (differential) manometers. The range covered by the nomograms is 16 to

27 ml. (total flask volume) for the Warburg type and 32 to 43 ml. for the Barcroft type. It has been published at a nominal cost to render it available to laboratories using manometric techniques where it will undoubtedly be most useful.

W. W. UMBRETT, Ithaca, N. Y.

**Annual Review of Biochemistry**, Vol. XV. Editor, J. MURRAY LUCK. Annual Reviews, Inc., Stanford University Press, California, 1946. xiii + 687 pp. Price \$5.00.

"Annual Reviews" have now become such an established and essential part of the armament of research workers in medical sciences that one now hardly dares to remember the days before these sound volumes appeared each year to encourage and invigorate the spirits of those who tend to wilt before the ever increasing flow of journals. The late war inevitably reduced the amount of literature available for or needing review each year and this effect is still apparent in the volume under review. The possibility that the expected post-war increase in the amount of literature, published and available for review, might inconveniently inflate the "Annual Review of Biochemistry" has been wisely anticipated by the decision of Annual Reviews, Inc., to publish a new series, the "Annual Review of Microbiology," the first volume of which is to appear in November 1947. Three articles in the present volumes of the "Annual Review of Biochemistry," namely, "Growth Factors for Microorganisms" (Snell), "The Biochemistry of Yeast" (Neuberg) and "Bacterial Metabolism" (Barker & Doudoroff), would find themselves in the new publication in any future year, while parts of others ("Biological Oxidations and Reductions"—Elliott, "Non-oxidative Enzymes"—Wynne, and "The Vitamins"—Dutcher and Guerraant, to mention but three) would accompany them. However much one may regret the increasing specialization of research interest which this further subdivision represents, the desirability of providing a new series of volumes to cover the rapidly growing field of Microbiology will be doubted by none. It is to be hoped that this mitosis will cause the parent unit to become less bulky and more easily manageable in future.

The division of the subject into more or less arbitrary sections, each of which is reviewed independently by an expert in the particular field of activity, inevitably leads to overlapping between the various articles. This is particularly noticeable in the present volume and if a master editor were to eliminate repetitive material its size could be greatly reduced. But, to this reviewer at least, it seems outstandingly desirable that the individual contributor should always be given a free hand in the treatment of the subject, and such freedom necessarily leads to overlapping. In any case, new material may need to be considered in a number of different relationships and the boundaries of the convenient but arbitrary sections will need to be crossed in numerous directions. Enzyme action lies at the basis of much of importance in biochemistry and it would be manifestly foolish to attempt to confine all consideration of enzymes within the two articles devoted to this subject in the volume under review.

When it is realized that many European journals published during 1945 were not accessible to the compilers of the present number, the potential increase in size in the post-war years becomes alarming. But as the literature to be surveyed grows and widens so does the indispensability of "Annual Reviews" become more certain. The titles of subjects which appear for review in this series for the first time in the current volume reflect new points of active interest. "Organic Insecticides," by Hoskins and

Craig, covers in a masterly fashion an interesting meeting ground of organic chemistry, botany, entomology and toxicology, which has yielded results of the greatest practical interest during the past three years, while "Inactivation and Detoxication of Pressor Amines" (Hartung) reviews in a modern manner evidence relevant to the classic problem of the relationship of structure to pharmacodynamic properties. The article by James on "The Respiration of Plants" is particularly welcome while "The Biochemistry of Teeth" (Leicester), the third of its kind, provides good support for its author's contention that the work in this subject is now based on a much firmer foundation than were the earlier studies.

"Annual Reviews" are a welcome example of international cooperation. Of the 21 articles in the present number, 13 are from authors in the United States, 4 come from Great Britain, 3 from Canada and one from Switzerland. The labor involved in ranging over such a wide field is great and the editors are to be congratulated on their success in this task. Since the previous volume appeared the death of Professor C. L. A. Schmidt, a member of the Editorial Board of the "Annual Review of Biochemistry" since its inception, and President of Annual Reviews, Inc., since 1936, has occurred. To such a colleague the reader of "Annual Reviews" owes much more than he probably realizes and it is fitting that an *In Memoriam* article concerning his life and work should appear in this volume.

Although he was writing on "The Biochemistry of Teeth," a happy sentence in Dr. H. M. Leicester's article might be applied to the position of biochemistry in general as revealed by the volume under review. "The process of integrating a very large quantity of previously unrelated data is now well under way, and holds much promise for the future."

F. G. YOUNG, London, England

**Organic Preparations.** By CONRAD WEYGAND, Professor at the University of Leipzig. Translated and revised from Part II of *Organisch-chemische Experimentierkunst*, Johann Ambrosius Barth, Leipzig (1938), by a group of collaborators. Interscience Publishers, Inc., New York, 1945. xiii + 534 pp. Price \$6.00.

This volume is a translation and revision of part II of Weygand's *Organisch-chemische Experimentierkunst*, Barth, Leipzig, 1938. As such, it is a welcome addition to any chemical library, and will find a place on the shelves of many individual organic chemists at or above the graduate student level. Published under license of the Alien Property Custodian, the book has been translated by a cooperating group.

In justifying their omission of parts I and III of the original, the publishers make the following comment:

"Part I, on materials and operations necessary for the execution of organic syntheses, deals mainly with German laboratory equipment, so that a literal translation would have limited value for the American reader. An adaptation of the text to American equipment would have necessitated a complete rewriting of this section, a task considered beyond the scope of a translation. Part III of the German book, on the chemical and physical analyses of organic compounds, is covered by a number of excellent American texts. A translation would thus have constituted a duplication we wished to avoid."

This reviewer, however, has found sections of both parts I and III of the original extremely useful, and feels that a better case for the omission of these sections can



be made on the grounds that their inclusion would have placed the book beyond the means of the average student. The high cost of the original was the principal disadvantage of an excellent book.

A further saving has been effected in the translation of part II by replacing the extensive literal quotations from "Organic Syntheses" by references to the individual and collective volumes of this work. In other respects, the translation follows the German closely, and the general discussion, choice of examples and documentation, all excellent, are the responsibility of Professor Weygand.

The organization of the book follows the now familiar system of Weygand, recently adopted by Theilheimer in his *Synthetische Methoden der Organischen Chemie*, which has as its basis the formation and fission of the various linkages of the carbon atom. The principal advantages claimed for the scheme are methodical presentation and flexibility. The most obvious disadvantage is the dispersal rather than the concentration of related topics. In the opinion of the reviewer, the success of a book following this system depends to a great extent upon the efficiency of the indexing, fortunately well done in this case.

The typography is tastefully designed with liberal use of small type for specific examples, and of italics and bold-face type for outline headings.

The book can be recommended heartily for use at the research level, and the translators and publishers are to be commended for a significant contribution to the literature of the organic laboratory.

MARSHALL GATES, Bryn Mawr, Pa.

**Spuren von Stoffen entscheiden über unser Schicksal. (Traces of Substances Determine Our Destiny.)** By EMIL ABDERHALDEN. Benno Schwabe and Co., Basel, Switzerland. Second Ed., 1946. 106 pp., 62 Figs. Price Sw. Fr. 6.00.

The "wonders of science" are frequent subjects for the popular writer. What is not so generally appreciated, however, is that these are, in good part, the wonders of nature which science has uncovered. The achievements of the last couple of decades in unraveling the complexities of animal physiology are indeed matters for congratulation, but the mechanisms for the control and balance of the organism, which have thus been elucidated, turn out to be wonderful beyond description. No embellishments, no journalese, are needed to make clear to the layman just how remarkable these are. A simple statement of the facts, shorn of unnecessary technical terms, should be enough. Even the physiologist or biochemist working in these fields seldom sees the wood for the trees, and a recapitulation of the whole astonishing story can hardly fail to stimulate him into a clearer visualization of the horizons which have been opened.

Hence Dr. Abderhalden, in this little book of popular science, has been content simply to recount some of the facts. The "traces of substances" which determine our fate are, of course, the vitamins and hormones, although at the end of the book the author, who has had a long and well-known career in enzyme research, discusses briefly some enzymes and coenzymes, as well as viruses. Throughout, he lays emphasis on the facts rather than on the achievements of science in elucidating them. Names of the major workers are, however, mentioned, though with a slight over-attention to the parts played by the Swiss and Germans. Starting with the conservation of

matter and general metabolism, we are soon switched into sexual physiology and the steroid hormones. Then come the pituitary, the thyroid and other glands and their activities. After this follows a review of the vitamins and brief mention of some of the other "trace" substances. Enough is said here and there about assay methods and about some of the key experiments, such as those of Berthold and of Hopkins, to give the reader a glimpse of the type of research involved. Perhaps more of this should have been included if the layman were to understand the methods, and more particularly the Herculean labors, of scientists. However, this reviewer at least would prefer such material to be omitted altogether rather than to be presented in the form of the improbable laboratory arguments, hunches and "human interest stories" which figure so prominently in the writings of other scientific popularizers.

It is evident that Abderhalden is fully alive to the remarkable character of nature's control mechanisms. Indeed, he is apt to wax teleological in his enthusiasm. Thus, after describing the hormonal relations of pregnancy he goes on (free translation):

"Now something else happens. The mammary glands must be prepared for their future function, that is, to nourish the newborn organism with milk. Up to now these have rested and, until onset of the first pregnancy, have never functioned. First everything must be prepared morphologically. The milk ducts develop. This takes place under the influence of the follicular hormone. The corpus luteum hormone (Progesterone) influences the development of the special secretory epithelium. Now all is in readiness. The only remaining requirement is a stimulus to the formation of milk. . . . A special hormone is forthcoming to bring the secretory activity of the milk glands into action. This is produced by the anterior lobe of the above-mentioned hypophysis and is called Prolactin."

Here and there a somewhat fresh idea is introduced. In emphasizing the essential inseparability of vitamins and hormones, Dr. Abderhalden points out that if we eat free Vitamin A in food it is truly a vitamin, but if it is produced in the liver from carotene it is to all intents and purposes a liver hormone. It is no harm to remind ourselves that terms like these imply utilitarian, rather than fundamental, distinctions. He might have made a similar point in regard to the role of thiamine in plants (in which it can be both a vitamin and a hormone) but the discussion does not include the hormone relations of plants.

As befits a simple presentation the language is on the whole simple. Except for an occasional lapse into a quarter-page sentence, the German is straightforward enough for those American graduate students who have worried their way through a few German papers. Indeed the book would be very useful in a course in scientific German. And as an indication of how other nations go about popularizing science it might well be studied by some of our own writers in this field.

KENNETH V. THIMANN, Cambridge, Mass.

**Trace Elements in Plants and Animals.** By WALTER STILES, M.A., Sc.D., F.L.S., F.R.S. 189 pp. Macmillan Co., 60 Fifth Ave., New York City, N. Y. Price \$2.75.

This small monograph forms a most timely addition to the literature of physiology, especially plant physiology. The term "trace elements" in the title is meant to indicate inorganic elements effective in the physiology of plants or animals in extremely small quantity. The term "trace element" is frequently used to designate such elements,

but the reviewer questions the appropriateness of the term. Some of the so-called trace elements are essential for the growth of higher plants in amounts which can often be more or less accurately estimated, and the magnitudes of the amounts required for optimum growth of the plant may be far greater than usually inferred by the term "trace." Other terms often employed for elements of this category are: micronutrient elements or minor elements. The latter is commonly used by the agronomist, but this term is also open to criticism, since the elements in question are as essential to plant growth as other inorganic elements required in relatively large quantity.

Stiles has prepared a comprehensive monograph in the sense that all the important general phases of the subject are considered. The review of literature is selective and more critical than most reviews on this subject. There are other publications in which a much more complete bibliography of investigations on the elements effective on plant growth in minute amounts is surveyed by presentation of disconnected abstracts, but the present book is especially valuable because it attempts to emphasize work which has added to our basic knowledge of this phase of plant and animal physiology. Many of the published articles are of only local or temporary significance.

The monograph is developed in such a way as to make it of great value to the student of plant nutrition in its purely scientific phases, as well as in the applied phases of concern to the soil scientist, horticulturist and agronomist.

While the monograph is not intended as a handbook of analytical methods, there is a useful discussion of the general types of analytical methods that are used for estimating in plant and animal tissues minute amounts of inorganic elements of physiological significance. Reference is made to colorimetric, spectrographic and polarographic methods, in part based on the author's own experience. The exacting methods of plant culture necessary for the study of the effects of the trace elements on plant growth are described.

This monograph does not have as one of its objectives the detailed review of biochemical processes in plant or animals, with extensive citation of chemical equations. Nevertheless, this volume is of great interest to biochemists insofar as it surveys physiological responses, especially of plants, to the trace elements, and attention is drawn to many important problems of biochemical nature awaiting exploration. The inadequacy of our present information on the functions of minute amounts of inorganic elements in the biochemical system of the plant is brought into focus, and the main facts of what is known are summarized rather for the general reader than for the specialized biochemist. Discussion of some interrelations of the trace elements in plants and animals will stimulate the interest of workers in animal nutrition.

Most of our knowledge of the relation of trace elements to plant growth has arisen in the past two decades, and has now come to have very important economic applications in agriculture. The author of this monograph has performed a distinctly useful service in preparing at this time a concise, well digested and well written review of this field of study, with citation of the principal key references to the literature, within the scope of the monograph.

D. R. HOAGLAND, Berkeley 4, Calif.

**Qualitative Analysis by Spot Tests.** By FRITZ FEIGL, Laboratorio da Producao Mineral, Ministerio da Agricultura, Rio de Janeiro, Brazil, formerly Professor of Analytical and Inorganic Chemistry, University of Vienna. Third English ed., trans-

lated by RALPH E. OESPER, Professor of Chemistry, University of Cincinnati. Elsevier Pub. Co., Inc., Amsterdam and New York, 1946. xiv + 574 pp. Price \$8.00.

Like the earlier books by Dr. Feigl, this text exemplifies the care, thought and thoroughness of the author. The expansion of the section on the working aids and general directions is a welcome addition. This reviewer, however, believes that inclusion of the theoretical portion which appeared in the first German edition, or even the shorter disquisition which appeared in *Ind. Eng. Chem., Anal. Ed.* 8, 401 (1936), or in the "Manual of Spot Tests" (1943), would have made this volume even more valuable.

The inclusion of the methods for detection of the members of the platinum group is a welcome addition, as is the employment of spot methods in preliminary procedures prior to complete analysis for inorganic substances in aqueous solution or suspension.

The section on the use of spot techniques in qualitative organic analysis has also been enlarged to include methods for the determination of oxygen, adjacent diketonic and methylene-ketonic groupings, secondary aliphatic and primary aromatic amines, together with the original work on the remaining organic groups, tests for which have been, in many cases, considerably expanded and revised.

Several Germanic idioms appear in the text, although these do not detract from its value to the biochemist, mineralogist, metallurgist, and chemical or sanitation engineer. The text, a classic in its field, is practically a "must" for the research library or for the busy analyst in the purely chemical or applied industrial fields.

WALTER A. HYNES, New York, N. Y.

**Penicillin: Its Practical Application.** Under the general editorship of SIR ALEXANDER FLEMING, Professor of Bacteriology in the University of London, St. Mary's Hospital, London. The Blakiston Company, Philadelphia, Pa., 1946, 380 pp. Price \$7.00.

It seems fitting and proper that Sir Alexander Fleming, who first described penicillin, should edit as well as contribute to a book on the general subject of penicillin and its practical application. This book contains an excellent summary of the history of the development of penicillin as well as a summary of the basic experimental and clinical experience accumulated by the investigators in Great Britain up until the time of its writing. Unfortunately, the book contains only two chapters by Sir Alexander Fleming: one on the history and development of penicillin and the other on the bacteriological control of penicillin therapy. These two chapters in themselves make the book useful and valuable to those interested in the general subject of chemotherapeutic and antibiotic agents. The chapters by Sir Alexander Fleming, like his writing in general, are presented in an interesting, orderly and concise fashion.

The book is divided roughly into two sections. The first section contains, in addition to the two chapters already mentioned, four additional chapters dealing with the chemistry, pharmacology and methods of administration of penicillin. The second section consists of twenty chapters dealing with the clinical application of penicillin. These chapters are written by outstanding clinicians and investigators in Great Britain. As one would expect in a situation where there are so many contributors, there occurs a certain amount of overlapping of material in various chapters. This is not especially objectionable since repetition in a sense increases emphasis on certain

points. An occasional chapter is rather brief and it appears as though the contributor grew tired of his assignment and as a result referred the reader to other publications for material which might well have been included in the manuscript.

The clinical section deals with the prophylactic use of penicillin and its use in general infections, bacterial endocarditis, thoracic infections, wounds and burns, as well as its use in orthopedic and plastic surgery. This section of the book also contains valuable information concerning the use of penicillin in certain specific diseases such as osteomyelitis, infections of the hands, and abdominal infections. The clinical section also contains material on the use of penicillin in obstetrics, sepsis neonatorum, infections of the brain and meninges, and venereal diseases. It also contains chapters on the use of penicillin in ophthalmology, otolaryngology and dermatology as well as in dental and oral infections and in certain animal diseases. A final chapter deals with the subject of penicillin and the general practitioner. Unfortunately, the contributor did not develop this chapter to the limit of its possibilities. Like some of the chapters mentioned previously, it suffers from brevity.

The book is interesting. It is easy to read. Each chapter contains adequate references to the subjects discussed and with few exceptions the contributors have selected the more important contributions for inclusion. Anyone interested in the subject of penicillin will find this book a useful and valuable addition to the library.

WALLACE E. HERRELL, Rochester, Minn.

*L'acide nicotinique, facteur de croissance pour "Proteus vulgaris."* (Nicotinic Acid, the Growth Factor for *Proteus vulgaris*.) By MADELEINE MOREL. 105 p. Masson and Company, Paris, 1943.

The studies reported in this monograph of the Pasteur Institute are a continuation of earlier investigations of Lwoff and Querido on the same subject, which in turn are based on the original work of Fildes. According to the author the strain *Proteus* "X<sub>1</sub>, Syrie" of the collection of the Pasteur Institute has many virtues, perhaps the most important being that its nutritional requirements and the response to nicotinic acid are very constant, so constant that it is not even necessary to carry out standard experiments in every series. No change in the properties of this strain was observed over a period of five years. The growth-promoting properties of related compounds and the ingredients of the medium were discussed extensively. The method for the determination of nicotinic acid has been evidently worked out to a stage which might be termed "foolproof." The examples of applications in the final chapter, however, are somewhat sparse and make it difficult to compare the method with other techniques. Other chapters review the specificity of nicotinamide for man and dog; chemical determinations; and the use of other microorganisms for assay of this vitamin.

The virtue of this brief monograph lies in the clear presentation of the subject outlined in the title. The history of the advent of nicotinic acid as seen by a microbiologist provides a stimulating variation from the usual presentation in which the mammalian requirement is emphasized. Whenever the author goes beyond the limits indicated in the title, the treatment is, of necessity, sketchy. The developments in this field have been so rapid that a new and extended edition would seem desirable. It is felt, however, that this monograph, even in its present form, will have to be studied by everyone concerned with the microbiological assay of nicotinic acid.

F. SCHLENK, Houston, Texas

# Some Physicochemical Properties of the Three Albumin Layers of the Chicken, Goose and Emu Egg

Vernon L. Frampton

*Cotton Research Committee of Texas, University of Texas, Austin, Texas*

and A. L. Romanoff

*Cornell University, Agricultural Experiment Station, Ithaca, N. Y.*

Received February 3, 1947

If one carefully opens a freshly laid egg and gently pours the contents into a petri dish, he will observe that a portion of the albumin flows out over the surface of the dish and that another portion of the albumin remains piled up about the yolk. These two portions of the albumin make up two morphologically distinct concentric layers of the white of the egg. The more fluid of these two portions, that is, the one which flows out over the surface of the petri dish, may be removed with a pipette, and it is designated in this report as the "outer layer" of the albumin. The more gelatinous portion is designated as the "intermediate layer." If one will now pierce the intermediate layer, after he has removed the outer layer from the dish, he will observe that the contents of the third layer—the "inner layer"—will flow out through the breach made in the intermediate layer, and it will also spread out over the surface of the dish. This third layer may also be removed with a pipette, and finally the intermediate layer may be removed from the yolk. (A fourth layer, the chalaziferous, a thin film which surrounds the yolk, can be gently separated along with the chalazae which are attached to the yolk at each pole.)

The chalazae and the chalaziferous layer of the albumin are deposited on the yolk in the fore part of the oviduct during the formation of the egg; the intermediate layer is deposited subsequently as the egg moves down the oviduct; the outer layer is deposited on the egg in the uterus before the shell formation; the inner layer is derived from the intermediate layer, and it is said by embryologists to be a "degradation

product" of the intermediate layer. Embryologists, of course, have long been familiar with the morphologically distinct layers of the albumen and have made several studies of their properties (1, 2, 3, 4, 5, 6, 7 and 8). Some of the data are summarized in Table I. The chemist, on

TABLE I  
*Physicochemical Properties of the Various Albumin Layers Of the Chicken Egg*

Observation	Albumin layer				
	Outer	Inter- mediate	Inner	Chalazi- ferous	Refer- ences
Ammonia (mg./100 cc.)	0.145	0.168	0.160		(2)
Ovomucin (% of total)	1.91	5.11	1.10		(3)
Ovoglobulin (% of total)	3.66	5.59	9.59		(3)
Ovalbumin (% of total)	94.4	89.2	89.3		(3)
Light transmission ( $I/I_0$ )	0.915	0.482	0.808		(4)
Mucin content (%)	0.145	0.591	0.227		(4)
Total solids (%)	11.6	12.5	14.6		(1)
Total solids (%)	11.7	12.2	12.9		(3)
Total solids (%)	11.4	12.2	13.1		(5)
Total solids (%)	10.7	12.9	13.8	15.8	(6)
Refractive index ( $[n]_D$ )	1.3529	1.3552	1.3582	1.3606	(6)
Refractive index ( $[n]_D$ )	1.3492	1.3566	1.3582		(7)
Density (d 25/25)	1.032	1.037	1.040		(8)
Proportional amount (%)	23.2	57.3	16.8	2.7	(6)

the other hand, has not taken cognizance of the morphology of the egg white, and in dealing with egg white, and in the isolation of the various components thereof, he has been content to mix the three layers together before proceeding with the isolation. It is probable that, as a consequence of this mixing, the several proteins which have been isolated from egg white, and which have been extensively studied, have been proven sooner or later to be mixtures of proteins. The classical example is that of egg albumin. Reference may be made to the work reported by Longsworth *et al.* (9). It will be demonstrated in a subsequent paper that two egg albumins may be crystallized from the inner layer and an egg albumin may be crystallized from each of the other two layers.

The separation of the three albuminous layers in the manner indicated is not quantitative. It is difficult, for example, to avoid contami-

nation of the outer layer with material from the intermediate layer, and to avoid contamination of the intermediate or inner layer with materials from either of the other two layers. The morphological structure of the intermediate layer, however, does permit an effective preparation of the three layers and, if the eggs are fresh, the contamination which does occur is not of great consequence. The data for the goose and the chicken eggs, which are included herewith, were obtained from eggs which were at most only a few hours old; the three layers were separately removed in the manner indicated above on the day the eggs were laid. The data were obtained from a composite of several eggs. The data for the emu egg<sup>1</sup> were obtained from a single emu egg which was about three days old. A comparison of some physico-chemical properties of the contents of the chicken, goose and emu eggs are presented in Table II. It will be noted that a gradient in the several properties is apparent as one proceeds inwardly from the shell.

In their work on the electrophoretic studies of the properties of the white of chicken eggs, Longworth, Cannan and MacInnes (8) diluted the whole white tenfold with acetate, phosphate and veronal buffers. We noted, as they did, that under these conditions there is an insoluble protein fraction, presumably ovomucin, which precipitates on dilution. They discarded the precipitate which was formed and conducted their study on the remainder of the protein. With our work an insoluble fraction was gotten from each of the layers from each of the eggs worked with. These fractions were observed, however, to be soluble in 1.6% sodium chloride solution, and in the preparation of the buffers used in this study, sodium chloride was added to the acetate buffer. At pH 4.45, and with each layer of albumin, there was a trace of material, apparently fibrous, which was difficultly soluble. The traces of material which remained in suspension after several minutes of stirring were removed on centrifugation before the scanning patterns were obtained with the electrophoresis apparatus.

The scanning patterns in Figs. 1, 2 and 3 for the chicken, the goose and the emu, respectively, were obtained after one hour of electrophoresis in 0.1 *M* acetate buffer at pH 4.45 and 1.6% sodium chloride, at a potential gradient of 1.7 volts/cm. Material for study was diluted tenfold. Comparisons, therefore, may be made relative to the concentrations of the proteins in each layer. The areas of the shadows obtained with the inner, the intermediate and the outer layers, respectively, are in the ratio 83:45:39 for the chicken, 63:70:76 for the goose and 74:76:67 for the emu. The patterns in Figs. 1, 2 and 3 are arranged in each figure with the pattern for each albumin layer im-

<sup>1</sup> The emu egg was kindly given to Dr. A. L. Romanoff by the Zoological Garden, Buffalo, New York.



TABLE II •

*Comparison of the Physicochemical Properties of the Contents  
of the Chicken, Goose and Emu Eggs*

Observation	Albumin layer			
	Yolk	Outer	Intermediate	Inner
Dry matter (%)				
emu	54.50	10.34	10.71	11.29
goose	55.90	—	12.9	—
chicken	51.40	10.70	12.85	13.72
Density (d 26/25)				
emu	—	—	—	—
goose	—	—	—	—
chicken	1.037	1.0315	1.0346	1.0369
Refractive index ( $[n]_D$ )				
emu	1.4202	1.3521	1.3532	1.3539
goose	—	—	—	—
chicken	1.4200	1.3529	1.3552	1.3582
pH (Beckman pH meter)				
emu	6.12	8.78	8.62	8.52
goose	5.84	—	8.67	—
chicken	5.97	7.98	7.91	7.76
Fat (% of wet wt. Soxhlet extn. with diethyl ether)				
emu	41.75			
goose	36.0			
chicken	31.6			

mediately below the other, with the dotted line drawn through the positions corresponding with the zero points. The conditions under which these determinations were made were as nearly identical as it was possible to make them, *e.g.*, the potential gradient, the pH and the quality of added electrolyte was the same in each case. One may, therefore, compare the relative mobilities of the protein components of each albumen layer. The evidence from these data is that no major protein components in any one layer may be identified with any major protein component in either of the other two layers.

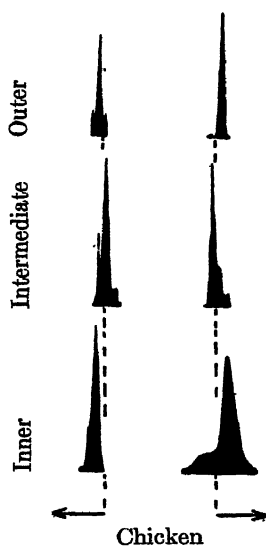


FIG. 1. Scanning Patterns Obtained with the Three Albuminous Layers of the White from Chicken Eggs.

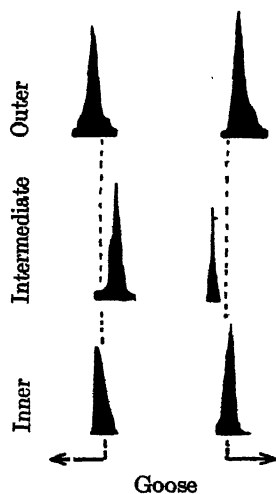


FIG. 2. Scanning Patterns Obtained with the Three Albuminous Layers of the White from the Goose Egg.

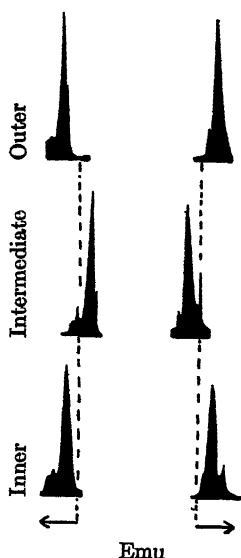


FIG. 3. Scanning Patterns Obtained with the Three Albuminous Layers of the White from the Emu Egg.

Another point of interest is that whereas the electrophoretic data obtained by Longworth *et al.* indicate the presence of seven protein components in the white of the chicken egg, the data separately obtained with the three layers at pH 4.45 indicate a minimum of 10 protein components.

#### SUMMARY

1. Some of the physicochemical properties of the contents of the three albuminous layers and yolk of the chicken, goose and emu egg are compared.
2. Electrophoretic scanning patterns obtained with each of the three albumin layers of the goose, chicken and emu egg indicate in each case that no major protein components in any one layer of egg albumin may be identified with any major protein component in either of the other two layers.
3. The data obtained with the chicken egg show at least ten protein components.

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# The Chemical and Biological Properties of Tryptic Digests of Casein and Lactalbumin

D. Bolling, R. J. Block and B. F. Chow

*From the Department of Physiology and Biochemistry, New York Medical College, New York, N. Y., and The Division of Protein Chemistry, The Squibb Institute for Medical Research, New Brunswick, N. J.*

Received December 26, 1946

## INTRODUCTION

In a previous paper (1) we have presented data to show the difference in the plasma protein regeneration properties of the two tryptic digests prepared from casein and lactalbumin. It was shown that a prolonged feeding of casein hydrolyzate to protein-depleted dogs resulted in an increase of both plasma albumins and globulins. On the other hand, the administration of the lactalbumin hydrolyzate to similar animals stimulated an increase of albumins but not globulins.

It is generally believed that protein or protein hydrolyzate can regenerate the plasma proteins only when all the essential amino acids are present in adequate amounts. It is, therefore, of interest to ascertain whether there exists any difference in the amino acid patterns of these two hydrolyzates, which can account for the differences in their biological properties. The analytical results of these two hydrolyzates for 10 essential amino acids as well as tyrosine and cystine are presented in this communication.

## EXPERIMENTAL

### *Preparation of Hydrolyzates*

A 10% solution of edible casein at pH 7.6 was digested with purified trypsin until 28-30% of the total polypeptide nitrogen was liberated as amino nitrogen. The mixture was then clarified by filtration and the filtrate was lyophilized. Similarly, a 10% suspension of lactalbumin (Borden 15-42) was digested with the same enzyme preparation until 35-40% of the total polypeptide nitrogen was liberated as amino nitrogen. The suspension was adjusted to  $\pm$ pH 5 and filtered. The clear filtrate was also lyophilized. These hydrolyzates were used for chemical analysis and were fed to

protein-depleted dogs (about 10 kg. in weight) at a daily dose of 0.35 g. of nitrogen/kg. body weight for about 30 days. The nitrogen balance experiments showed that 80-90% of the nitrogen fed was retained in the body.

### RESULTS

From the analytical results given in Table I, it can be seen that both hydrolyzates contain all the essential amino acids. The lactalbumin

TABLE I  
*Amino Acid Analysis of Casein and Lactalbumin Hydrolyzates.*

Amino acids	Casein hydrolyzate	Lactalbumin hydrolyzate	Method
	<i>Per cent</i>	<i>Per cent</i>	
Nitrogen <sup>a</sup>	16.2	14.8	Dumas
Arginine <sup>b</sup>	4.3	4.1	Microbiological and Kossel
Histidine <sup>c</sup>	3.1	2.1	Microbiological and Block
Lysine <sup>d</sup>	7.3	9.2	Microbiological and Block
Tryptophan <sup>e</sup>	1.4	2.1	Millon-Lugg
Phenylalanine <sup>f</sup>	5.5	5.1	Kapeller-Adler
Methionine <sup>g</sup>	3.5	2.4	McCarthy-Sullivan
Threonine <sup>h</sup>	3.9	4.3	Shinn-Nicolet
Leucine <sup>i</sup>	9.1	10.8	Microbiological
Isoleucine <sup>j</sup>	8.1	7.4	Microbiological
Valine <sup>k</sup>	7.9	6.3	Microbiological
Tyrosine <sup>l</sup>	2.8	2.7	Millon-Lugg
Cystine <sup>m</sup>	0.4	2.0	Folin

<sup>a</sup> Niederl and Niederl, *Org. Quant. Micro Analysis*, 2nd Ed., 79-100. John Wiley, 1942.

<sup>b</sup> Stokes, J. L., Gunness, M., Dwyer, I. M., and Caswell, M. C., *J. Biol. Chem.* **160**, 35 (1945).

<sup>c</sup> Dunn, M. S., Camien, M. N., Shankman, S., and Rockland, L. B., *J. Biol. Chem.* **159**, 653 (1945).

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<sup>e</sup> Lugg, J. W. H., *Biochem. J.* **31**, 1423, (1937). Millon, M. E., *Compt. rend.* **28**, 40 (1949).

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<sup>h</sup> Shinn, L. A., and Nicolet, B. H., *J. Biol. Chem.* **138**, 91 (1941).

<sup>i,j,k</sup> Shankman, S., *J. Biol. Chem.* **150**, 305 (1943); McMahan, G. R., and Snell, E. E., *J. Biol. Chem.* **152**, 83 (1944).

<sup>l</sup> Lugg, J. W. H., *Biochem. J.* **31**, 1423 (1937). Millon, M. E., *Compt. rend.* **28**, 40 (1949).

<sup>m</sup> Folin, O., and Looney, J. M., *J. Biol. Chem.* **51**, 421 (1922).

hydrolyzate contains significantly higher amounts of lysine, tryptophan and cystine. On the other hand, it contains less methionine and valine. Amino acid analyses by the same methods (see Table II) have also been

TABLE II

*Amino Acid Analysis of Purified Albumin and Globulin Fractions from Dog Plasma*

Amino acids	Albumin fraction	Globulin fraction
Nitrogen	15.2	13.8
Arginine	5.8	5.8
Histidine	3.1	2.7
Lysine	9.6	5.2
Tryptophan	1.7	2.6
Phenylalanine	6.9	5.8
Methionine	1.7	1.9
Threonine	4.0	5.4
Leucine	10.9	7.9
Isoleucine	2.2	3.9
Valine	7.6	6.0
Tyrosine	5.4	4.8
Cystine	4.3	2.1

made on plasma albumins and plasma globulins isolated from normal dog plasma, to find out whether dog albumins<sup>1</sup> or globulins are particularly rich in certain amino acids and, therefore, the synthesis of these proteins may have specific requirements of certain amino acids, which the hydrolyzates must supply.

The results given in Table II demonstrate that the albumin fraction contains significantly higher lysine, cystine, leucine, and valine, but less tryptophan than the globulin fractions.

Certain growth factors, like streptogenin, which has been demonstrated to play an important role in promoting the growth of bacterial organisms, mice and rats, may play a part in synthesis of plasma protein, even though it has not been demonstrated to be an important factor in dog or man. It is, therefore, of interest to determine the streptogenin contents of the two hydrolyzates. The average results of our determination (3) on several samples of hydrolyzates fed to our experimental dogs are: the casein hydrolyzate equals 5 units/g.; the lactalbumin hydrolyzate equals 4 units/g. Wilson's liver extract No. L-20 was used as reference standard (1 unit/g.). These results indicate that the

<sup>1</sup> The separation of albumins and globulins was effected by using 40% alcohol at pH 4.8 and 1°C.



samples of the casein hydrolyzate used for our experiments contained only a slightly higher amount of streptogenin than the lactalbumin hydrolyzate.

The growth efficiency and the biological values of these two hydrolyzates have also been determined. It was found that the lactalbumin hydrolyzate had a biological value of 1.0 for normal dogs and growth efficiency of 2.7. The biological value of the casein hydrolyzate<sup>2</sup> was 0.80 and its growth efficiency was almost 2.2. A calculation of the expected biological value in terms of nitrogen retention may be made from the amino acid composition, according to a formula derived by Mitchell and Block (4). This yields a figure for lactalbumin hydrolyzate of 0.86, and for casein digest of 0.75. These figures being lower than those activities observed may indicate that the actual utilization of the lactalbumin hydrolyzate is higher than was to be expected on the basis of its amino acid content, *per se*.

#### DISCUSSION

The nutritive value of protein or protein hydrolyzates can be assayed in a number of ways, biologically by nitrogen retention, growth efficiency,<sup>3</sup> plasma protein regeneration, lactation, *etc.*, and chemically by amino acid composition. It is generally believed that if a protein hydrolyzate contains all the essential amino acids in adequate amounts, it will promote growth, repair tissues and regenerate proteins. It is thus obvious that the nutritive value of a protein or mixture of proteins for any biological function or combination of functions is limited by the relative proportions of the essential amino acids contained in it, *i.e.*, those amino acids that cannot be synthesized by the animal at a sufficiently rapid rate from any substances present in the usual diets. Mitchell and Block (3) have found good agreement between the nutritive value of proteins as determined by feeding experiments and that which could be calculated from the limiting indispensable amino acid as estimated by chemical analyses. However, there are instances, as with the hydrolyzates studied, in which the biological value of a protein or protein mixture and its chemical (essential amino acid) rating do not agree. Various suggestions have been made to account for

<sup>2</sup> The casein hydrolyzate was obtained from E. R. Squibb & Sons.

<sup>3</sup> The authors wish to express their gratitude to Dr. Black of E. R. Squibb & Sons for making the growth efficiency tests.

this discrepancy, among which is that decreased digestibility of a portion may result in slower adsorption of certain amino acids and this result in a lower biological value than would have been achieved if all the potentially available amino acids had been presented at the proper time (4, 5, 6). Other factors, such as streptogenin (2), increased ease of digestion, *etc.*, may also play a role in enhancing or decreasing the nutritive value of protein foods. It is also possible that the various types of the polypeptide residues in a hydrolyzate are important factors in conditioning various specific effects.

The casein and lactalbumin hydrolyzates differ most significantly in their ratios of cystine to methionine. Since, of the nitrogen retained, less than 5% was utilized for the synthesis of plasma protein, it is unlikely that the inability of dogs fed with lactalbumin hydrolyzate to induce the production of globulins is due to any limiting factor of any amino acids fed. However, it is possible that after the primary demand for the essential amino acids to synthesize tissue proteins was met, the remaining essential amino acids in the lactalbumin hydrolyzate may be inadequate for the synthesis of plasma globulins. Experiments are now in progress to ascertain whether lactalbumin hydrolyzate supplemented by amino acids of which it is deficient, can promote the stimulation of globulins.

### SUMMARY

Lactalbumin hydrolyzate prepared by tryptic digestion has been found to have a higher biological value and growth efficiency than similar digests of casein. These two hydrolyzates also differ in their ability to stimulate the production of the plasma protein in dogs previously depleted in proteins. The difference in the plasma protein regeneration properties can not be attributed entirely to the slight difference in the essential amino acid content of these two hydrolyzates.

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## Isolation and Properties of Blood Group-Specific Substances from Horse Stomachs

Louis A. Kazal, Aeme Higashi, Rose Brahinsky,  
Millard DeYoung and L. Earle Arnow

*From the Department of Biochemistry, Medical Research Division,  
Sharp and Dohme, Inc., Glenolden, Pa.*

Received January 15, 1947

### INTRODUCTION

The addition of group A- and B-specific substances to universal donor (group O) blood for the purpose of neutralizing the anti-A and anti-B agglutinins prior to transfusion (1) has stimulated recently a greater interest in these serologically active polysaccharides. The group A-specific substance has been isolated from a variety of sources, such as hog gastric mucin (2, 3, 4), hog stomachs (5), various peptones (6), commercial pepsin (2, 7), urine (8), horse saliva (9), and various human tissues (10, 11, 12, 13). On the other hand, the group B-specific substance has been isolated chiefly from the tissues of group B individuals, for example, gastric juice (14), erythrocytes (10, 11), pseudo-mucinous ovarian cysts (12), and salivary and gastric mucin (13). A polysaccharide from *Ascaris suum* capable of reacting with anti-A and anti-B agglutinins has been reported (15). It is not always possible or convenient to obtain these raw materials for the isolation of the B substance. Therefore, the discovery of the existence of relatively large amounts of a B factor in the stomach and other glandular tissues of the horse (16, 17) has offered a more promising source of this polysaccharide, and has made possible the large scale preparation of solutions of group A- and B-specific substances for the conditioning of group O blood for transfusions (16).

In this paper we wish to describe our investigations<sup>1</sup> on the isolation of the B factor from horse stomachs.

<sup>1</sup> We are greatly indebted to Dr. Ernest Witebsky and Dr. Niels C. Klendshoj, of the Buffalo General Hospital, and to Dr. J. A. Leighty, of the Eli Lilly Research Laboratories, for disclosing the unpublished results of their experiments on the isola-

## EXPERIMENTAL

*Determination of Group A- and Group B-Specific Activity*

Group-specific activity was determined by the quantitative inhibition of isoagglutination method described by Witebsky (14). This method was modified<sup>2</sup> to employ (a) a diluted human typing serum with a constant concentration of anti-A and anti-B agglutinins, (b) a 10-minute instead of 15-minute period of incubation of specific substances with the diluted serum, and (c) 0.1 ml. instead of 0.2 ml. of a 1% red cell suspension. The degree of agglutination resulting from the mixtures of specific substance, serum, and erythrocytes was observed after incubation for 15 minutes at room temperature, followed by centrifugation for 1 minute at 1,500 r.p.m.

The concentration of typing serum to be employed in the inhibition test was determined by the usual technic for obtaining agglutination titers. The next to the highest dilution of serum showing complete agglutination (4+) of group A or B erythrocytes was the dilution of serum employed in the inhibition of agglutination determination. Group A and B red cells were obtained either from blood collected in 4% sodium citrate solution (1 vol. citrate + 9 vol. blood), or from blood collected without an anticoagulant. In the latter case a portion of the clotted blood was squeezed through a strainer, and taken up in saline. The red cells in both instances were washed at least once with an equal volume of saline before preparing the 1% suspensions for the tests. If a number of determinations were made throughout the day, fresh 1% suspensions of red cells were prepared frequently from the washed, concentrated stock cells that were kept at 5°C. This precaution had to be taken because dilute suspensions sometimes lost their sensitivity within a few hours.

Three-fold dilutions of solutions of specific substances (1 mg./ml.), or of saliva or enzymatic digests (pH 7), were prepared in saline for the inhibition of agglutination determinations. Early in this investigation samples of desiccated group A and group B substances were set aside as controls. A solution of both substances (1 mg. of each /ml.) was prepared and held in the frozen state at -20°C. This control solution was included with each set of determinations of unknowns as a check on any variations in the sensitivity of the erythrocytes, the strength of the agglutinating sera, etc.

*Methods of Isolation*

The methods used by Goebel (6) and by Witebsky and Klendshoj (14) for the isolation of specific substances from neopeptone and gastric juice, respectively, were adapted to the isolation of group specific substances from horse stomachs.

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tion of the B factor from whole horse stomachs and for technical advice in the early phases of this investigation.

Our thanks are due for the valuable assistance of Mr. Henry Rock, Glandular Products Department, Sharp and Dohme, for the preparation of some of the specific substances from large amounts of starting material.

<sup>2</sup>Dr. Ernest Witebsky kindly supplied us with the detailed description of this method.

The fresh tissues were solubilized by enzymatic digestion, in most instances by autolysis at pH 2, taking advantage of the presence of the gastric enzyme in the mucosa. Whenever the enzyme-containing portion of the mucosa was used, it was not necessary to add commercial pepsin to solubilize the tissue; thus in most instances the specific substances subsequently isolated from such autolyzates did not contain any of the group A-specific activity present in commercial pepsin (7). All autolyzates were precipitated with alcohol to obtain the specific substances. Some of the preparations were deproteinized by the method of Sevag (18) in somewhat the same manner as described by Goebel and by Witebsky and Klendshoj, while others were deproteinized by tryptic digestion. In the latter case the autolyzate of the stomach tissue was digested with trypsin and the specific substances were isolated from the digest by alcoholic precipitation.

A typical example of each procedure will be described here.

*Procedure No. 1 (Employing the Method of Sevag for Deproteinization)*

13.6 kg. of tissue were ground, suspended in 38 l. of tap water, acidified with concentrated HCl to pH 2, and allowed to autolyze at 50°C. for 19-21 hours in a temperature-controlled glass-lined tank.

The viscous autolyzate was heated to 85°C. and filtered through several layers of absorbent cotton to remove a small amount of lipid-like material. Three volumes of anhydrous alcohol<sup>3</sup> were added, and the precipitated specific substance was allowed to settle out overnight. The gummy precipitate (No. 1) was collected in a Sharples or cup centrifuge after siphoning off most of the supernatant, and then dissolved in 1% sodium chloride solution by extracting the precipitate with aliquot portions of the saline solution until complete solution was effected. The solution (28-52 l.) then was buffered at pH 6.8 with 14% granular sodium acetate and sufficient glacial acetic acid to obtain the desired pH. The pH was maintained below 7 by the addition of glacial acetic acid while the acetate was being dissolved, since there was some evidence from other experiments that exposure to an alkaline pH produced some loss of activity. The specific substance was precipitated (No. 2) from this buffered solution with 2.5 volumes of anhydrous alcohol.

The 2nd precipitate was redissolved and reprecipitated in the same manner as the first, except that it was dissolved in 14-28 l. of saline. A buffered (pH 4.6) solution of the 3rd precipitate was filtered through Reeve Angel No. 202 paper by gravity, and then deproteinized by the method of Sevag, using 1 volume of solution and 0.25

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<sup>3</sup> The precipitation of the group-specific substance from the digest by 3 vol. of alcohol was complete. The alcohol-soluble fraction, recovered by removing alcohol by vacuum distillation followed by dialysis, and drying from the frozen state, was devoid of any group-specific activity.

volume of a chloroform-butyl alcohol mixture (5 + 1). The mixture was shaken (24-95 hr.) until the supernatant gave a negative tungstic acid-biuret test. Three volumes of alcohol were added to the deproteinized solution. The precipitate (No. 4) was dissolved in 6-11 l. of distilled water, and again precipitated with 3 volumes of anhydrous ethanol. A few ml. of glacial acetic acid and/or 10% sodium chloride solution were added to induce more complete precipitation. The last precipitate was dried with alcohol and ether, or acetone, or a solution of it was dried *in vacuo* from the frozen state.

In the preparation of HS-13-A and HS-13-B, the glandular gastric mucosae from 47 stomachs were autolyzed individually, and the titer of each autolyzate determined separately. Thirteen highly active autolyzates (average B titer 1-367) were pooled, and then subjected to alcoholic precipitation essentially as described in procedure No. 1. This preparation was labeled HS-13A. Eight other autolyzates from this group (average B titer 1-40) were processed in a similar manner, and the isolated product labeled HS-13B.

### *Procedure No. 2 (Digestion with Trypsin)*

An autolyzate of fresh tissue, prepared as described above, was adjusted with 40% sodium hydroxide solution to pH 6.5. The neutralized solution was digested with 5.8 g. of trypsin (Wilson 1-300 or Difco 1-250)/kg. of fresh tissue at 40-45°C. for 20 hours. Toluene was added to prevent putrefaction.

The tryptic digest was heated to 85°C., cooled, and filtered by gravity through Reeve Angel paper No. 202. One *per cent* sodium chloride (wt./v.) was dissolved in the digest, followed by the addition of 3 volumes of anhydrous ethanol. The alcoholic precipitate so formed was subjected to 3 additional alcoholic precipitations from filtered aqueous solutions: twice with 2.5 volumes of ethanol from buffer-saline solution (pH 6.5) of volumes 13 and 7 l., respectively, and once with 3 volumes of ethanol from 5 l. of distilled water solution at pH 4-5 (adjusted with glacial acetic acid). The last precipitate was dried by washing with alcohol and ether, or else its solution was dried *in vacuo* from the frozen state. The buffer-saline solutions were prepared as described in procedure No. 1 for the solution of precipitates No. 1 and No. 2. However, in contrast to procedure No. 1, all the precipitates were dissolved at a temperature of 45-50°C. to hasten solution and all such solutions were clarified by filtration through Hormann D-2 or Republic K-3 pads. Otherwise the technics were the same as in procedure No. 1.

Procedure No. 2 was modified slightly for the isolation of group-specific substances from horse gastric mucin<sup>4</sup> and from trypsin (Wilson's 1-300). For the preparation of HSGM-1 50 g. of horse gastric mucin dissolved in 2.5 l. of saline were autolyzed, digested with trypsin (50 mg./g. mucin) and then precipitated several times with alcohol following the technic of procedure No. 2. The trypsin-derived substance was isolated in the same manner as was HSGM-1, except that autolysis at pH 2 was eliminated, and the trypsin solution was allowed to digest itself.

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<sup>4</sup> Prepared by the Wilson Laboratories, Chicago, Ill.

### *Analytical Methods*

Total nitrogen was determined by the semimicro Kjeldahl method. Samples were digested in concentrated sulfuric acid containing dissolved Se and in the presence of potassium sulfate, mercuric oxide, and copper sulfate. The technic of Cavett (19) was modified for the distillation of  $\text{NH}_3$  into boric acid (20). Reducing sugar (glucose) was determined by the Shaffer-Hartmann method as modified by Somogyi (21) using 0.5 *N* sulfuric acid hydrolyzates (6 hrs.) of the polysaccharides. The relative viscosities of 0.5% solutions of polysaccharide in distilled water were obtained at 37°C. with an Ostwald viscosimeter. A Schmidt-Haensch polarimeter was employed for the determination of specific rotation. pH determinations were made with a glass electrode (Beckman pH meter).

The biuret test was made on 1% solutions of the polysaccharide and also on tungstic acid precipitates obtained by mixing 5 ml. of solution with 1 ml. of 2/3*N* sulfuric acid solution, followed by 1 ml. of 10% sodium tungstate. In the latter test, the precipitate was collected by centrifugation, drained well and, dissolved in 0.5 ml. of 5% sodium hydroxide solution. One *per cent* copper sulfate solution was added dropwise to develop the biuret color.

### *The Activity of Saliva and Gastric Mucosa of the Horse*

Some observations on the type and titer of horse saliva and of digests of gastric mucosa were made to determine whether or not the presence and potency of the B factor of the gastric mucosa of horses could be ascertained by testing the saliva before the animals were slaughtered.

Saliva<sup>5</sup> (2-8 ml.) was collected by maintaining a small piece of thoroughly washed DuPont synthetic sponge in the mouth of the horse for approximately 10 minutes. It was heated in a boiling water bath for 15 minutes to destroy the blood group enzymes (22), and was then centrifuged. Procedure No. 1, described above and modified to handle smaller quantities of tissue, was used to prepare pH 2-autolyzates of washed, whole gastric mucosa, which subsequently were neutralized to pH 7 with 6 *N* sodium hydroxide solution. The salivas and digests were tested for group-specific activity by the inhibition of agglutination technic.

The results of these studies showed that the distribution of group-specific types of saliva was different from that of horse stomachs. In a group of 11 horses, the saliva of 5 belonged to group A (46%), 2 to group B (18%), and 4 to group AB (36%). No salivas belonging to group O, the latter being characterized by the absence of group A and B activity, were encountered. Four of these salivas showed only traces

<sup>5</sup> Drs. B. J. McGroarty and W. J. Mathey, of Sharp and Dohme, kindly collected the horse saliva used in this investigation.



of group A or B activity, or of both; the remainder possessed group A titers of 1:1 to 1:243 and group B titers of 1:1 to 1:9. On the other hand, the digests of 21 gastric mucosae were typed as group AB. As in the case of the salivas, some of these gastric linings contained only traces of group-specific activity, while others showed group A and B titers as high as 1:729. In two of these digests such small traces of group A activity were found (a 3 + reaction with the undiluted digest) that the possibility of finding a gastric lining with pure group B activity seemed promising at one time.

In some instances, it was possible to test the saliva and the digest of the same horse. These results, presented in Table I, illustrated the lack

TABLE I

*A Comparison of the Type and Titer of Saliva, the Gastric Mucosal Digest, and the Isolated Specific Substance in the Horse*

	Group specificity			Titer**					
				Saliva		Digest*		Specific substance	
Horse No.	Saliva	Digest*	Specific substance	A	B	A	B	A	B
5958	A	AB	AB	trace	0	trace	1:1	trace	1:1
5959	A	AB		1:27	0	1:243	1:1		
7937	AB	AB	AB	1:3	trace	1:1	1:27	1:1	1:27
7928	A	AB	AB	1:9	0	1:3	1:9	1:3	1:9
5451	AB	AB		trace	trace	1:3	1:81		
6289	AB	AB	AB	trace	1:1	trace	1:9	trace	1:9

\* An autolyzate (pH 2) of the entire gastric mucosa.

\*\* The dilution of saliva, digest, or a solution (1 mg./cc.) of isolated specific substance that produced complete inhibition of isoagglutination of human A or B erythrocytes.

of agreement between the group type and titer of saliva and of digests of gastric mucosa in some animals. In those instances, where both factors were present in the saliva and the gastric mucosa, it would not have been possible to predict the potency of the B factor in gastric mucosa from the saliva tests. The activities of the specific substances isolated from this group of digests paralleled that of the digests themselves.

*Group B Activity in the Glandular and Non-Glandular  
Regions of Gastric Mucosa .*

The gastric mucosa of the horse easily can be differentiated anatomically into two regions (23): the fundic gland and pyloric gland regions representing the glandular portion of the mucosa, and the esophageal region representing the non-glandular region. The former was observed to be a smooth, mucous-covered tissue of a dark red (fundic) and tan (pyloric) color. It represented approximately  $\frac{2}{3}$  of the total weight of the gastric lining. The esophageal region was a white, stratified, non-mucoid epithelium, closely adherent to the muscular wall of the stomach, and separated from the glandular region of the mucosa by a well-defined "limiting" or "cuticular" ridge. The glandular, in contrast to the non-glandular, region easily was separated from the muscular wall of the stomach. The group-specific activity of these two regions was studied.

Individual autolyzates were prepared from the glandular and from the non-glandular regions of a number of horse gastric mucosae, using the conditions specified in procedure No. 1. Since very little, if any, digestion occurred in the non-glandular region due to the absence of gastric enzymes, 10 mg. of pepsin (Difco 1:15,000)/g. of fresh tissue were added to digest the ground suspension of the non-glandular tissue. As a control measure, this same amount of pepsin also was added to some of the glandular tissues. All digests were neutralized to pH 7 with 40% sodium hydroxide solution, and their group B titers determined. Specific substances were isolated from each digest by the method outlined in procedure No. 1.

These experiments are summarized in Table II. In general, the digests of the glandular portions of the gastric linings were much more active than those of the non-glandular portions. Furthermore, it was possible to isolate substances with greater activity and in large quantities from the glandular region. Since the greater portion of the group B activity resided in the glandular tissue, which easily could be separated from the gastric muscular wall, the glandular portion of the gastric lining appeared to be the best starting point for the isolation of group specific substances. The group A titers of these preparations and digests, which were quite high, have not been reported here, because the greatest portion of this activity was derived from the group A

TABLE II  
*Group B Activity of Digests and Isolated Specific Substances of the  
 Glandular and Non-Glandular Portions of Horse Gastric Mucosa*

"Glandular" mucosa				
Preparation	Fresh tissue per stomach	Titer†		Yield of specific substances
		Digest	Specific substance	
10-4	285	1:27	1:27	235
10-9*	335	1:27	1:9	230
10-11*	437	1:81	1:27	172
10-12	346	1:243	1:27	197
10-13*	483	1:243	1:81	155
"Non-glandular" mucosa				
10-4*	103	1:9	1:3	56
10-9*	162	0	1:1	86
10-11*	183	1:27	0	83
10-12*	157	1:9	0	83
10-13*	178	1:9	1:1	62

† Dilution of the digest or of a stock solution of specific substance (1 mg./cc.) that produced complete inhibition of isoagglutination of group B erythrocytes.

\* Digest prepared with 10 mg. pepsin/g. fresh tissue.

substance present in the commercial pepsin that was used in the preparation of the digests.

#### *Group-Specific Substances Isolated from Horse Stomach Tissue*

Preparations of the group specific substances that were isolated from whole stomach, from non-selected and selected glandular portions of the gastric mucosa, and from horse gastric mucin, are described in Table III. The selected mucosae were obtained by preparing individual digests of gastric linings. In a group of 47 linings, 35% gave group B titers of almost zero to 1:3; 36%, 1:9 to 1:27; and 28%, 1:81 to 1:729. HS-13A was isolated from the highly active or 28% group; HS-13B, from the 36% group.

All the isolated specific substances were amorphous, white powders that, with one exception (P-HS-2, from whole stomach), dissolved slowly in water or saline to form either opaque or slightly opalescent

TABLE III  
Group Specific Substances from Horse Stomachs

Isolation procedure	No. 1 Sevag				No. 2 Trypsin		
Preparation No.	P-HS-2	P-HS-1	HS-13B	HS-13A <sup>b</sup>	P-HS-4	P-HS-4D	HS-GM-1
Source	Whole stomachs	Gastric mucosae	Selected mucosae	Selected mucosae	Gastric mucosae	Dialyzed P-HS-4	Gastric mucin
Number of stomachs	50	50	8	13	100		18
Wt. of material processed, kg.	57.5	13.6	2.4	3.5	27.2		0.05
Yield, per cent	0.2	0.08 <sup>a</sup>	0.16	0.16	0.31		9.6
Group B activity, $\gamma^*$	33.3	11.1	11.1	0.41	1.2	1.2	1.2
Group A activity, $\gamma^*$	>100	>100	33.3	33.3	3.7	3.7	3.7
Total nitrogen, per cent†	13.2	10.2	9.6	7.5	6.7	7.3	7.7
Ash, per cent	4.2	8.7	6.3	8.8	22.7	9.7	12.0
Reducing sugar (glucose), per cent†	21.7	35.4		37.6	51.2		43.9
Relative viscosity, 37°C., 0.5%	1.71	2.21	3.68	3.49	2.13	2.55	1.99

\*  $\gamma$  of specific substance showing complete inhibition of isoagglutination of human erythrocytes in the quantitative test.

† Corrected for moisture and ash.

<sup>a</sup> Low yield due to loss of some material during deproteinization.

<sup>b</sup>  $[\alpha]_{30}^D = +20^\circ$ .

solutions (HS-13A and HS-13B) at 1% concentration. The pH of these solutions varied from 6.1 to 7.0.

It was possible in the case of solutions prepared from some substances isolated by the trypsin procedure (No. 2) to change the property of opaqueness to opalescence merely by adjusting the pH to 4-5 with acetic acid. Upon readjustment to pH 7 with alkali, the solutions became cloudy due to the formation of an inactive flocculent precipitate which now easily could be removed by centrifugation. This treatment greatly simplified the filtration and sterilization of solutions intended for the conditioning of group O blood.

The specific substances obtained by both procedures were not precipitated from 1% solution by 10% sulfosalicylic or trichloroacetic acids. In some cases slight precipitation was produced at a concentration of 1.5% tungstic acid. The tungstic acid precipitate was biuret-negative (tungstic acid-biuret test) except for P-HS-2. Apparently, the protein present in P-HS-2 could be removed from solution by adsorption with an excess of Lloyd's reagent following the procedure of Meyer (3). The treated material failed to give a positive tungstic acid-biuret test, and was as active as, or slightly more active than, the untreated preparation.

## DISCUSSION

The group-specific substances isolated from horse gastric mucosa were capable of inhibiting the isoagglutination of group A and group B human erythrocytes, and, accordingly, may be classified as group AB, as previously reported by Witebsky (17). A tremendous variation in the group A and B activity of horse gastric mucosae, somewhat analogous to the variation of group A activity in hog stomachs found by Kabat (30), was observed among different horses. In some instances only traces of group A and/or group B activity were found in the digests of horse gastric mucosae or in the specific substances isolated from these digests. Attempts to predict the presence and potency of the B factor by testing the saliva of the horse before slaughtering were fruitless due to the lack of specificity, as well as to differences in the activity, of the saliva and of the gastric mucosa of the same horse (Table I). Witebsky (17) has reported similar qualitative and quantitative differences for these tissues of the horse.

The isolation of a specific substance with a high group B activity by procedure No. 1 was dependent upon the proper selection of gastric mucosal tissue. This was evident from the observations (1) that most of the group B activity was confined to the glandular region of the gastric mucosa, (2) that the non-glandular region contributed a substance with little or no activity (Table II), which, by its presence, diluted the activity of the more active substances, (3) that individual "glandular" digests could be segregated into essentially inactive, moderately active, and highly active groups on the basis of their group B titers. The most potent group-specific substance (HS-13A, Table III) was isolated from a pooled batch of highly active glandular digests, having group B titers of 1-81 to 1-729. Complete inhibition of agglutination of 0.2 cc. of a 1% suspension of group B cells by the concentration of anti-B agglutinins used in the test was produced by 0.41  $\gamma$  of this substance. The group-specific substance isolated by Witebsky (14) from human (secretor) gastric juice appears to have been somewhat more active than HS-13A. However, the conditions under which the tests were performed were not identical in the two cases.

Starting with less active digests or with non-selected gastric mucosae or whole stomachs, it was possible to isolate less active specific substances (Table III). The relatively constant yields and variable activities, and the isolation of essentially inactive substances in approxi-

mately the same yield from some individual mucosae (unpublished data), suggested that the less potent preparations were mixtures of active and of inactive or less active substances. The trypsin procedure was not used to isolate specific substances from similar types of starting material. However, it was possible to isolate very active preparations by this method from previously supposed poor sources of starting material (Table III). Horse gastric mucin was an excellent source of an active group B factor, much like hog gastric mucin for the group A factor. However, while the yield from the dry mucin was high, the overall yield from fresh tissue was considerably smaller.

A complete chemical analysis of the group-specific substances has not been attempted<sup>6</sup> since, in their present state, undoubtedly they are not pure substances. There was a rough parallelism between nitrogen content and activity, suggesting the presence of foreign nitrogenous substances in the less active preparations. The specific substances isolated by the trypsin procedure possessed unusually high ash contents that could be reduced approximately to one-half of the original value by exhaustive dialysis. Table III summarizes the chemical properties thus far studied for specific substances isolated from horse stomachs.

All the specific substances isolated from horse gastric mucosae or mucin gave weak biuret reactions. Whether the weak biuret reaction, which repeatedly has been reported in the literature for purified specific substances, was an indication of the presence of peptide bonds in the molecule or merely of the presence of protein impurity is not clear. It is difficult to evaluate the purity of isolated substances in terms of the presence or absence of protein, since experimental evidence favors the existence of amino acids (4, 25, 26, 27, 28) in group-specific substances, perhaps as polypeptide chains in the molecule.<sup>6</sup> The use of the tungstic acid-biuret test, in conjunction with active anaphylaxis, did not clarify the situation, since all preparations tested were anaphylactogenic in

<sup>6</sup> Preparation No. 960-P-HS-3 (comparable to P-HS-4) contained 14.3% glucosamine and 37% of reducing sugar, calculated as glucose. The total nitrogen was 6.0%. The following amino acids were present, in the amounts (g./100 g. of substance) indicated in brackets: glycine (0.6), valine (0.6), isoleucine (0.2), proline (0.9), glutamic acid (2.8), phenylalanine (0.3), tryptophan (0), histidine (0.6), lysine (0.7), aspartic acid (1.6), serine (2.0), tyrosine (0.2). For similar data on hog group A substance cf. reference (29). We are indebted for these determinations to Dr. E. Brand from the Department of Biochemistry of Columbia University.

guinea pigs, even when no precipitation was produced by tungstic acid. The activity of the group-specific substances isolated by either procedure did not approach the order of activity of the group A substances reported in the literature. That the trypsin method was capable of isolating very active material was demonstrated by the isolation of the group A substance from hog gastric mucin by the trypsin procedure as well as by the 90% phenol-10% ethanol fractionation method of Morgan and King (4).<sup>7</sup> The isolated substances were equally active, 0.05  $\gamma$  producing complete inhibition of agglutination of group A red cells, and, so far as it was possible to make comparisons they appeared to have approximately the same activity as Morgan and King's preparation. It would appear that the low order of activity of the group A and B substances isolated from horse gastric mucosa was not the result of poor isolation technics.

All group-specific substances isolated from the horse displayed some group A activity. In the case of the trypsin method of isolation, a trace of this activity may have been introduced by the trypsin used in this procedure. A substance<sup>8</sup> with only a trace of group A activity, but with a high content of nitrogen and ash, and a low content of reducing sugar, was isolated from trypsin in a yield of 4.5% by procedure No. 2. By calculation it was present in approximately 9% concentration in the group-specific substances of the horse. Obviously, further purification necessitates the elimination of this contaminating material.

#### SUMMARY

Group-specific substances were isolated from whole stomachs, gastric mucosa and gastric mucin of the horse by methods involving enzymatic digestion and precipitation with alcohol. Deproteinization was effected by the Sevag procedure and by digestion with trypsin.

The isolated specific substances possessed both group A and B activity; group B activity always was greater than group A activity. However, individual gastric mucosae in the form of autolyzates ex-

<sup>7</sup> A detailed report of our data on the activity and the chemical and physical properties of the group A substances from hog stomachs, gastric mucosa, and gastric mucin has not been reported since such preparations already have been adequately described by Morgan and King (4), Kabat (29) and others.

<sup>8</sup> The substance derived from Wilson's trypsin (1-300) contained 14.1% total nitrogen (corrected for moisture and ash), 22.8% ash and 22.9% reducing sugar (calculated as glucose).

hibited a wide range of group A and/or B activity, with group A activity predominating in a few instances.

There was no relationship, either qualitative or quantitative, between the group-specific activity of the gastric mucosa and of the saliva in the same horse.

The glandular region of the gastric mucosa, in contrast to the non-glandular one, contained the greatest concentration of, and the most active, group-specific substances. The most active group-specific substance was isolated from mucosae selected for their high activity. However, by the use of trypsin, material almost as active was obtained from non-selected specimens of mucosa.

The trypsin method was capable of isolating the group A substance from hog gastric mucin in a state apparently as active as that reported in the literature. However, trypsin contained a substance, resembling the group-specific substances in some respects but having only very weak group A activity, which presumably was present as a contaminant in the isolated group-specific substances.

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# Effect of Insulin Hypoglycemia on Brain Glucose, Glycogen, Lactate and Phosphates \*

Norman S. Olsen and J. Raymond Klein

*From the Departments of Psychiatry and Biological Chemistry,  
University of Illinois College of Medicine, Illinois  
Neuropsychiatric Institute, Chicago*

Received January 22, 1947

## INTRODUCTION

It has been reported that hypoglycemia evoked by insulin is accompanied by decreases in brain glucose (1) and glycogen (1, 2), but by no change in lactate or phosphocreatine (3). However, under different experimental conditions brain lactate has been found to decrease during hypoglycemia (4).

In the present investigation the effect of intramuscular injection of insulin upon brain glucose, glycogen, lactate, pyruvate, and phosphates was determined in paralyzed cats maintained with artificial respiration. The effects of hypoglycemia on brain were followed by means of its changes in electrical activity.

## EXPERIMENTAL

Complete details, concerning the preparation of the animals for the experiment and the methods of assay, have been reported (5, 6). The following details will suffice for present orientation. Mature, healthy, female cats, kept on a standard diet for at least two weeks, were paralyzed with dihydro- $\beta$ -erythroidine hydrobromide<sup>1</sup> and maintained with artificial respiration. Electrical activity of cortex and heart were followed by means of Grass amplifiers and ink writers. At various intervals after intramuscular injection of crystalline zinc insulin, 5 units/kg. body weight, half into each front leg, the brains were frozen by pouring liquid air on the exposed skull. A sample of arterial blood was taken simultaneously. The control series was obtained in a similar manner, all conditions being similar except for the injection of insulin.

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\* Aided by a grant from the Rockefeller Foundation. A preliminary report has been presented before the meeting of the Association for Nervous and Mental Diseases, New York, December, 1946.

<sup>1</sup> Supplied by Merck and Co., Inc., through the courtesy of Dr. D. F. Robertson.

The frozen brains were removed, ground to a fine powder while still frozen and assayed for glucose, glycogen, lactate, pyruvate, phosphocreatine, adenosine di- and triphosphates, and inorganic phosphate.

## RESULTS

The results obtained for the control and experimental series are given in Table I. The data indicate that the administration of insulin was followed in most instances by a decrease in brain lactate, pyruvate, glucose, glycogen, phosphocreatine, adenosine triphosphate, and acid-labile phosphate (the sum of that derived from adenosine di- and triphosphate) and an increase in adenosine diphosphate. The concentration of inorganic phosphate in "severe coma" appears to be increased, however, the variations in the control level being sufficiently large to preclude unequivocal interpretation. These changes were accompanied by decreases in plasma lactate, pyruvate, and glucose.

The changes in the electrical activity of heart, which were similar to those previously described (7), were insufficient to imply a degree of hypoxia that would account for the changes in composition of brain. Further, the low levels of plasma and brain lactate contraindicate the presence of anoxia.

The usual sequence of changes in electrical activity of brain is illustrated in Fig. 1. The type of activity at the time of freezing is indicated in Table I. There was no marked relation between the degree of chemical change and duration of change in electrical activity. Relatively little chemical change occurred during the early "hypoglycemic" period; marked chemical changes occurred after the onset of "coma."

## DISCUSSION

The present work indicates that insulin hypoglycemia leads in brain to a decrease in the concentrations of glucose, glycogen, lactate, pyruvate, phosphocreatine, and adenosine triphosphate and increases in the concentrations of adenosine diphosphate and inorganic phosphate. That Kerr and Ghantus (3) did not note changes in concentration of lactate and phosphocreatine may have been due to their experimental conditions. Their animals were given amytal at some time after onset of hypoglycemic symptoms and the brains were frozen when anesthesia had been induced. In the present investigation the brains were frozen immediately after the appearance of the desired changes as noted by the electroencephalograph.

TABLE I  
*Effect of Insulin Hypoglycemia on Brain and Blood Metabolites of Cats*

Five units of crystalline zinc insulin/kg. body weight was injected intramuscularly, one-half of the total dose into each front leg. Plasma was obtained from an arterial blood sample taken at the time the brain was frozen. The kind of electrical activity of brain at freezing is illustrated in Fig. 1. The control data, which have been reported previously (5, 6), are means and standard deviations. The number of assays for each substance in the control series is given in brackets. All results are given in mM/1,000 g. or ml.

Brain										Plasma		
Minutes— insulin to freezing	Brain activity at freezing	Lactate	Pyruvate	Glucose	Glycogen	Phospho- creatine	Adenosine triphos- phate	Adenosine diphos- phate	Inorganic phosphorus	Lactate	Pyruvate	Glucose
Controls												
	Control	2.2 ±0.5 (13)	0.22 ±0.11 (10)	4.2 ±1.1 (11)	6.8 ±1.1 (9)	2.4 ±0.5 (14)	1.3 ±0.5 (10)	1.1 ±0.7 (10)	4.7 ±1.8 (14)	2.2 ±1.2 (12)	0.17 ±0.02 (12)	12.0 ± 2.9 (12)
Experimental												
150	Hypoglycemic	1.8	0.15	1.6	7.1	1.1	1.0	1.7	7.7	1.6	0.23	4.0
240	Hypoglycemic	2.4	0.16	0.3	6.1	2.0	1.5	1.4	5.0	0.8	0.07	1.9
240	Hypoglycemic	2.6	0.31	0.2	6.0	1.6	0.2	2.2	6.9	0.7	0.09	1.2
210	Hypoglycemic	2.4	0.07	0.2	6.0	1.4	0.5	1.4	4.3	0.8	0.05	1.2
240	Hypoglycemic	0.6	0.10	0.2	5.9	1.6	2.5	0.1	8.6	—	—	1.0
120	Hypoglycemic	2.2	0.18	1.8	5.2	1.9	0.8	2.0	3.3	2.3	0.20	5.7
120	Coma	0.3	0.09	0.1	3.2	1.7	—	—	5.9	0.5	0.10	1.5
180	Coma	—	0.05	—	3.2	1.5	0.0	1.8	3.8	0.3	0.05	0.8
180	Coma	0.7	0.10	0.4	2.3	1.1	1.3	1.1	5.2	—	0.08	1.8
195	Severe Coma	0.8	0.06	0.1	2.9	1.2	0.0	1.4	7.9	0.4	0.06	0.6
330	Severe Coma	1.2	0.08	0.4	1.0	0.8	0.0	2.1	11.4	0.8	0.11	0.9
420	Severe Coma	2.6	0.07	0.2	1.1	0.6	0.0	2.6	—	1.0	0.06	0.6

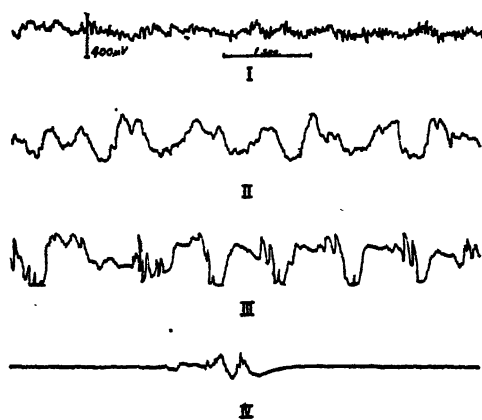


FIG. 1. Electrical activity of brain during insulin hypoglycemia. The electrical activity represented was recorded by means of Grass amplifiers and ink writers from screw electrodes in the frontal region of the skull. The transition from control activity to that appearing during hypoglycemia was gradual. The activity illustrated represents successive phases of the transition. The first line (I) in the figure shows the control pattern. The second (II) indicates activity which in the intact cat would accompany definite hypoglycemic symptoms. The activity represented in the third line (III) in the intact cat would accompany coma. Severe coma would give activity similar to that in the last line (IV).

In mammals, administration of insulin can lead to increases in glycogen content of liver and muscle, but not of brain (3). In contrast, insulin given to frogs leads to increases in brain glycogen (8). Epinephrine, when injected or when released from the adrenal medulla by reflex action during hypoglycemia, causes a decrease in muscle glycogen (9). Administration of epinephrine has no effect on brain glycogen (1, 3). Consequently, the decrease in brain glycogen during hypoglycemia can not be attributed to secretion of epinephrine.

The decreased cerebral blood flow and cerebral arteriovenous differences in glucose and oxygen during insulin hypoglycemia (10) indicate a decreased rate of glucose metabolism, induced by inadequate rate of supply of glucose to brain. The changes in composition of brain during hypoglycemia induced by insulin are attributable to the decreased rate of glucose metabolism rather than to direct effect of insulin on brain.

The symptoms of hypoglycemia manifested by the central nervous system are attributable to depression of cortex and consequent release

of lower structures. It seems reasonable to assume that the depression of cortex is caused by the decreased metabolism.

Functional activity of tissue is thought to be supported by the breakdown of adenosine triphosphate, resynthesis of which is effected by oxidation of glucose, while phosphocreatine aids in maintaining the concentration of adenosine triphosphate. The changes in functional activity and composition of the cerebrum during hypoglycemia may be correlated as follows. The decreases in glucose and glycogen are due to an inadequate rate of supply of glucose to the brain. The resulting decrease in rate of glucose metabolism accounts for the lowered level of lactate, pyruvate, and adenosine triphosphate in brain. The decrease in adenosine triphosphate, in turn, may be the limiting factor in the functional activity of the cerebrum.

#### SUMMARY

Hypoglycemia, induced by intramuscular injections of insulin, causes in brain a decrease in concentration of glucose, lactate, pyruvate, glycogen, phosphocreatine, adenosine triphosphate and acid-labile phosphate and an increase in concentration of adenosine diphosphate. Decreases in plasma lactate and pyruvate accompany the hypoglycemia. There is no marked correlation between the degree of chemical change in brain and the duration of a change in electrical activity produced by the hypoglycemia. However, the chemical changes become more marked as change in electrical activity progresses.

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# Evaluation of Torula Yeast Protein in the Life Cycle of the Rat

A. A. Klose and H. L. Fevold

*From the Western Regional Research Laboratory,<sup>1</sup> Albany, Calif.*

Received January 27, 1947

## INTRODUCTION

Previous reports from this laboratory (1, 2) have shown that yeast protein lacked sufficient methionine to produce optimum growth when fed at a 13.5% crude protein level to young rats. Indeed, diets containing 40% yeast, equivalent to 21% crude protein, gave lower growth rates than did casein at 11% crude protein. This deficiency was found to be common to samples of both torula yeast and brewers' yeast, each behaving in essentially the same manner.

The nutritional equivalence of the protein of torula and brewers' yeasts has also been indicated in a report on poultry feeding by Temperton and Dudley (3), in the feeding of swine and cattle (4), in the work of Hock (5), and in the work of Hock and Fink (6) with rats. Correspondingly, complete analyses of the essential amino acids (Block and Bolling (7) and Edwards *et al.* (8)) in the two types of yeast have been in good overall agreement. Asenjo (9), as judged by one experiment, reported that brewers' yeast had a 25% higher biological value than torula yeast. In a second experiment the two yeasts were fed at inadequate protein levels; brewers' yeast produced a better growth than torula, but even so, it was less than one-third of optimum growth rate.

In the work reported here, the value of torula yeast protein fed at a 20% crude protein level was measured during growth, reproduction, and lactation by means of *ad libitum* feeding experiments with albino rats, extending through four successive generations. Also, measurements were made on the growth of mothers and litters fed torula and brewers' yeast diets during the 21-day lactation period only. In both sets of experiments, the supplementary effect of methionine and cystine on yeast protein was determined.

<sup>1</sup> Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.



## EXPERIMENTAL

The samples of yeast used in this study were the same as those described in our earlier reports (1, 2), namely, torula yeast (*Torulopsis utilis*) grown on molasses, and a commercial brewers'-type yeast grown on a hop-free grain medium. On a dry basis, the two yeast samples contained 53 and 44% crude protein, respectively. A commercial sample of casein containing 84% crude protein was used as a reference protein.

*Experiment I.* Uniform groups of 40-day old rats—9 males and 9 females per group—were selected from the laboratory stock colony and fed the diets listed in Table I. The resulting average gains and feed

TABLE I  
*Composition of Diets for Experiment I*  
(in per cent)

Ingredient	Diet			
	1	2	3	4
Basal mixture <sup>1</sup>	57	57	57	57
Corn starch-sucrose (50-50)	5.5	5.3	5.3	23
Torula yeast (Molasses)	37.5 <sup>2</sup>	37.5	37.5	—
Casein (commercial)	—	—	—	20 <sup>3</sup>
Yeast extract (50% methanol)	—	—	—	≈5
Cystine	—	0.2	—	—
Methionine	—	—	0.2	—

<sup>1</sup> The basal mixture contained, as *per cent* of the complete diet, corn starch 20.5, sucrose 20.5, cottonseed oil 10, salt mixture (McCollum's No. 185 plus trace elements) 4, cod liver oil (U.S.P.) 2, vegetable oil concentrate containing 40% tocopherols 0.05; and as mg./100 g. of complete diet, choline chloride 50, thiamine hydrochloride 0.2, riboflavin 0.5, pyridoxin 0.2, calcium pantothenate 2.5, and niacin 1.0.

<sup>2</sup> Equivalent to 20% crude protein ( $N \times 6.25$ ).

<sup>3</sup> Equivalent to 17% crude protein ( $N \times .638$ ).

consumption over a 42-day period were recorded and are presented in Table II. At about 95 days of age, the rats were mated within groups. The males were then sacrificed and their organs weighed. No significant differences between groups were found with respect to weight and appearance of the liver, kidneys, spleen, adrenals, testes, or seminal vesicles. The breeding record of the females and the growth data on the litters produced, which we shall call generation 2, are given in Table III. Of generation 2, the rats of group 2 (yeast + cystine) alone were

TABLE II  
*Growth of Young Rats on Yeast Protein*  
Experiment I<sup>1</sup>

Group	Protein supplement	Average gain and standard error g./rat/day		Feed consumption g./rat/day		Efficiency g. gain/g. protein eaten	
		Males	Females	Males	Females	Males	Females
1	Torula yeast (20% crude protein)	2.10 ± 0.10	1.41 ± 0.06	10.7	8.8	0.98	0.80
2	Torula yeast + 0.2% cystine	2.58 ± 0.16	1.73 ± 0.08	10.3	8.3	1.25	1.04
3	Torula yeast + 0.2% methionine	2.67 ± 0.14	1.64 ± 0.08	10.9	8.2	1.23	1.00
4	Casein (17% crude protein)	4.10 ± 0.13	2.27 ± 0.09	14.7	10.7	1.64	1.25
5	Stock diet	3.45 ± 0.18	2.10 ± 0.14	17.1	13.9	—	—

<sup>1</sup> 42 day period, 9 males and 9 females/group, 98 g. average initial weight.

saved. At about 100 days of age, these rats were mated, producing litters of generation 3, which in turn were mated at 100 days to produce generation 4. Generations 2, 3, and 4 were fed diet 2 only. The breeding

TABLE III  
*Breeding Record of Rats (Gen. 1) Raised to Maturity on Yeast Diets*  
*and Growth of Their Litters (Gen. 2)*  
Experiment I

Group	Diet	Average weight of females at mating	Number of females per group	Number of litters born	Average number of young per litter	Per cent survival of young at 21 days	Average weight of young at 21 days	Growth of young (Gen. 2) from 31st to 82nd day of age		
								Average gain g./rat/day	Feed consumption g./rat/day	Efficiency g. gain/g. protein eaten
1	Yeast	157	8	6	7	12	18	No survivors after 43 days of age		
2	Yeast + Cystine	164	9	8	7	61	18			
3	Yeast + Methionine	156	9	7	6	75	24	2.09	11.0	0.95
4	Casein	186	9	9	10	86	30	2.86 Discarded	10.7	1.57
5	Stock	181	9	9	8	96	27			

and growth data are summarized in Table IV. Litters from generation 2 were not separated from the mothers until 40 days of age. All other litters were weaned at 21 days.

*Experiments II and III.* Pregnant six-month old females, fed the stock diet of Purina Dog Chow, cod liver oil, and liver prior to parturition, were placed on the diets described in Table V during the lactation period. Females having litters of 8-10 rats were used. All litters were

TABLE IV  
*Breeding Record of Generations 2 and 3 on Yeast Protein Diet,  
 Supplemented With 0.2% Cystine*

## Experiment I

Female No.	Weight of female at mating (g.)	Number of young in litter	Number of survivors in litter at 40 days—Gen. 3 21 days—Gen. 4	Average weight of young at 21 days (g.)	Average gain in weight of young after weaning over period of 42 days—Gen. 3 31 days—Gen. 4 g./rat/day
Generation 2, Producing Litters of Generation 3					
2a	145	5	3	—	1.6 (average for all rats)
2b	145	7	5	—	
2c	152	3	3	—	
2d	138	2	2	—	
Generation 3, Producing Litters of Generation 4					
3a	162	9	9	23	1.6
3b	146	5	5	25	2.4
3c	165	7	7	24	2.1
3d	174	7	6	33	2.5

reduced to 8 rats for the test. The resulting weight changes in mothers and litters are given in Table V.

## DISCUSSION OF RESULTS

Referring to Table II, the rate of growth obtained with yeast protein at a 20% crude protein level is in agreement with our earlier results (2). Supplementing the yeast at this level with 0.2% cystine or 0.2% methionine resulted in a significantly higher growth rate, still below that of casein, however.

The responses of our experimental animals to yeast diets are not in complete agreement with those of other workers. We found no abnormalities in weight or macroscopic appearance of the liver and other organs in male rats fed the yeast diet for 74 days, or in females on the yeast diet for periods exceeding 100 days. Hock and Fink (6) reported a high mortality and excessive liver damage throughout a 90-day period in which rats were fed a yeast diet. A supplement of cystine somewhat improved this condition. Himsworth and Glynn (10) de-

scribed a massive hepatic necrosis, which developed after a variable latent period of several weeks in rats on yeast diets, and which could be prevented by a methionine supplement. The absence of liver damage in our rats may have been due to a prolonged latent period, or more probably to the high level of yeast, which may furnish a preventive level of methionine.

TABLE V

*Effect of Yeast Protein Diets on Weight of Rats During Lactation Period  
(in grams)*

Diet Experiment II 3 mothers, 24 young/group	Average weight of mothers		Average weight of young	
	At birth of young	20 days after	At birth	20 days after
Diet 1 of Exp. I (Torula Yeast)	244	182	6	19
Diet 1 plus 0.2% Methionine	229	241	6	36
0.3% Cystine Stock Diet	226	242	6	31
Experiment III 5 mothers, 40 young/group	2 days after birth	21 days after birth	2 days after birth	21 days after birth
Diet A <sup>1</sup> (Brewers' yeast)	234	197	7.2	24.5
A plus 0.3% Methionine	232	235	7.0	40.8
A plus 0.3% Cystine	222	230	6.6	35.6
Diet B <sup>2</sup>	216	212	7.7	39.7

<sup>1</sup> Diet A was composed of basal mixture 57, brewers' yeast 43, and provided 19% crude protein.

<sup>2</sup> Diet B was composed of basal mixture 57, corn starch 10.5, sucrose 10.5, brewers' yeast 2, and casein 20. This diet provided 18% crude protein.

Table III presents the breeding record of rats maintained on the experimental diets from 40 days of age. The number of successful pregnancies and the size of litters were normal. However the death, in a state of inanition, of 88% of the young on diet 1 during the lactation period is strong evidence of the inadequacy of the yeast at a 20% crude protein level to support lactation. The survival and rate of growth of the young on the cystine- and methionine-supplemented yeast were suboptimum but strikingly superior to those of the un-supplemented yeast group.

When four females of generation 2 on diet 2 (yeast-cystine) were mated to litter mates, rather small litters were born (Table IV). Of the survivors of these litters, four females (generation 3) were mated successfully and produced average size litters (generation 4) which grew as well as had generation 1 on the same diet.

We may conclude from the data of Experiment I that the yeast protein alone at a 20% crude protein level is inadequate to sustain the normal life cycle. Supplemented with methionine or cystine, it gives adequate but suboptimum results.

The results of Experiments II and III (Table V) show that the use of yeast protein alone in a lactation diet results in the loss of weight for the mother, and a weaning weight for the young of less than two-thirds of normal. Yeast diets furnishing 19% crude protein, supplemented with either 0.3% cystine or 0.3% methionine, were demonstrated to be adequate for the lactation period. Weaning weights were normal for mothers and litters.

There was essentially 100% survival of the litters in Experiments II and III, in contrast with Experiment I. This probably reflects the better overall state of nutrition of the mothers of Experiments II and III prior to parturition.

#### SUMMARY

A yeast protein diet furnishing 20% crude protein was found to be adequate for the growth of rats at a suboptimum rate over extended periods, and for successful matings and pregnancies. It was inadequate for the lactation period, and death of the litters occurred. Addition of methionine or cystine to the yeast diet resulted in an increased, but still suboptimal, rate of growth and permitted a normal lactation period. In the case of mothers fed adequate stock diets prior to parturition, the supplementation of a yeast lactation diet with methionine or cystine greatly increased the weaning weight.

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# Gramicidin Derivatives. I. Preparation; Hemolytic and Bacteriostatic Properties

Otto Schales and Godfrey E. Mann

*From the Chemical Research Laboratory of the Alton Ochsner Medical Foundation, New Orleans 15, La.*

Received February 26, 1947

## INTRODUCTION

In 1939 Dubos (1, 2) obtained from cultures of *Bacillus brevis* a protein-free concentrate which showed considerable antibacterial activity. Most of the antibiotic effect was subsequently found to be due to the presence in these concentrates of two alcohol-soluble polypeptides, tyrocidine and gramicidin, which were both isolated in crystalline form (3, 4).

Tyrocidine is bactericidal for a variety of gram positive and gram negative organisms and has been found to inactivate completely the oxidation-reduction systems of susceptible cells (5). The compound has a pronounced effect on cell membranes, similar to that of cationic detergents, ultimately resulting in lysis (1, 6). Its antibacterial activity is reduced markedly in presence of albumin or plasma proteins (4, 6), a property which, together with its marked toxicity, removes this substance from the list of compounds suitable for systemic use in the treatment of infectious diseases.

Gramicidin is bacteriostatic for a variety of gram positive organisms (with the exception of spore-forming bacilli) and for *gonococci* and *meningococci*. Other gram negative organisms are not inhibited by this compound. Unlike tyrocidine, the substance is equally effective in presence or absence of proteins and seems to interfere with only a few specific metabolic functions of susceptible organisms (5), so that its presence does not terminate abruptly metabolic activity and life.

Chemical data obtained thus far (6-13) suggest that gramicidin is a cyclic polypeptide with a molecular weight of about 2800. No free amino or carboxyl groups have been found to be present (9), but it is



concluded from acetylation experiments (9) that the molecule contains two hydroxyl groups. Gramicidin apparently is composed of 24 units, which have been accounted for as follows: 6 as L-tryptophan, 6 as D-leucine, 5 as D- and L-valine, 3 as L-alanine, 2 as glycine and 2 as aminoethyl alcohol. Aminoethanol actually has been isolated from gramicidin hydrolyzates by Synge (7), and seems to be present in a linkage less resistant to acid hydrolysis than the average peptide bond. It is difficult to visualize a cyclic peptide without free amino and carboxyl groups containing only the constituents enumerated above. This problem has not yet been solved and requires further analytical work. The data of Hotchkiss and Dubos (8) on the elementary composition of gramicidin suggest a ratio of 148 C atoms/30 N atoms. The 24 units mentioned contain a total of 144 C and 30 N atoms, so that the possibility of the presence of "extra-carbon" in form of a nitrogen-free constituent has to be considered. So far, search for such a compound in gramicidin hydrolyzates has been negative (7, 9).

Gramicidin shares with tyrocidine the ability to hemolyze red cells. This hemolytic effect of gramicidin, although considerably slower than that of tyrocidine, appears to account for a good deal of its toxicity for animals (14) and has prevented the intravenous use of this bacteriostatic agent. Lewis *et al.* (15) reported recently the significant finding that a substance obtained on treatment of gramicidin with formaldehyde (methylol gramicidin) had only 1/12 of the hemolytic activity of the parent compound, whereas the activity against *Staph. aureus* had remained unchanged. The properties of methylol gramicidin have been confirmed in general in this laboratory, using slightly different testing procedures. Furthermore, gramicidin has been subjected to various types of chemical reactions which have resulted in a number of derivatives. Several of the new compounds, described in this report, have had only a fraction of the hemolytic and toxic properties of the starting material but have displayed considerable bacteriostatic activity.

## EXPERIMENTAL

### A. Determination of Antibacterial Activity

The organisms used were *Staphylococcus aureus* (A.T.C.C. No. 6538) and *Streptococcus Lancefield* H69-D5, a non-pathogenic group D hemolytic streptococcus. Bacteriostatic activity was measured turbidimetrically. Fresh cultures (18-24 hr.) grown in medium II of Schmidt and Moyer (16) were diluted with sterile medium.

The dilutions used were  $10^{-5}$  for *Staph. aureus* and  $10^{-3}$  for *Strep. Lancefield*. Ten ml. portions of these diluted cultures were pipetted into sterile Pyrex test tubes (18.5 mm. diameter) which contained 0.1 ml. alcoholic solution of the test substance in various concentrations. The mixtures were incubated for 18.5 hr. at  $37^{\circ}\text{C}$ . The turbidity in each tube was read (after careful mixing) against sterile medium with a Lumetron Photoelectric Colorimeter 402-E, Filter M-575. The extinction values thus obtained corresponded to a certain percentage of full growth which was read from calibration curves. The percentage of growth observed was then plotted against the corresponding concentration of antibacterial agent and from the resulting curves the concentration required for a 50% inhibition was determined (see Figs. 1 and 2). In

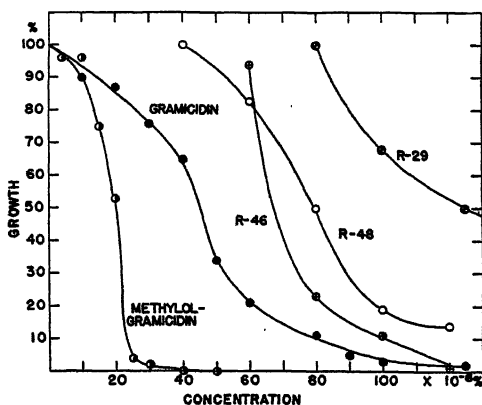


FIG. 1. Inhibitory effect of gramicidin and of some gramicidin derivatives on the growth of *Staph. aureus* No. 6538.

Culture dilution:  $10^{-5}$ ; Incubation time: 18.5 hr.

all assay series were included two "blank" tubes, each of which contained 10 ml. diluted culture and 0.1 ml. alcohol. The average turbidity of these two tubes represented full growth. With *Staph. aureus* the extinction values of these "blank" tubes were about 85-90% of those obtained in absence of alcohol. *Strep. Lancefield* gave slightly higher turbidities in presence of alcohol with extinction values about 3-5% above those found if no alcohol was present.

The calibration curves mentioned above were prepared by reading the turbidity of various dilutions (with sterile medium) of "blank" tubes and plotting extinction values against dilution, i.e., against the corresponding percentage of full growth. Typical calibration curves relating turbidity and percentage growth are shown in an earlier communication from this laboratory (17). One mg. of gramicidin was found to inhibit growth by 50% for 18.5 hr. in about 2 l. of dilute *Staph. aureus* culture, whereas the same quantity caused 50% inhibition for 18.5 hr. in about 30 l. of dilute *Strep. Lancefield* culture.

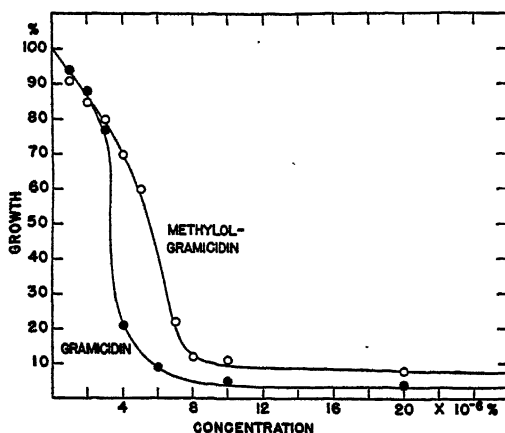


FIG. 2. Inhibitory effect of gramicidin and methylol gramicidin on the growth of *Strep. Lancefield* H69-D5.

Culture dilution:  $10^{-3}$ ; Incubation time: 18.5 hr.

### B. Determination of Hemolytic Activity

The method used for the determination of the hemolytic activity of gramicidin and its derivatives is similar to that described by Dimick (18) for the quantitative determination of tyrothricin. Hemolytic activity was determined by measuring the rate of disappearance of turbidity in washed suspensions of human red cells in the presence of selected concentrations of the test substances. The decrease in turbidity was followed with a Lumetron Photoelectric Colorimeter 402-E, Filter M-660. Oxalated human blood was diluted with 17 volumes of isotonic NaCl solution (0.87%) and centrifuged. The supernatant was removed and the remaining red cells were washed once more with 17 volumes of NaCl solution. The cells were then suspended in isotonic NaCl solution, bringing the total volume to ten times that of the original blood. This stock blood suspension could be stored in the refrigerator for 24 hr. without any significant change in its response to the hemolytic agents investigated here.

For each test series, 10 ml. of a stock blood suspension was diluted to 200 ml. with isotonic NaCl solution and mixed carefully to ensure uniformity of suspension. Ten ml. portions containing the cells from 0.05 ml. blood were pipetted into colorimeter tubes, and the initial *per cent* transmission values were determined against distilled water. To each tube was then added a 0.1 ml. portion of various alcoholic solutions containing known concentrations of the substances to be tested, and the tubes were inverted 4 times for thorough mixing. Usually the alcoholic solutions were made up so that the final concentrations of the test substances in the erythrocyte suspensions were those required for 50% inhibition of *Staph. aureus* (C50% S. A.). Transmission readings were taken at 10 min. intervals after addition of the alcohol solution, the tubes being inverted gently 4 times before each reading. A "blank" tube which

contained 0.1 ml. alcohol and 10 ml. of the red cell suspension showed only negligible changes in transmission and no hemolysis for periods up to 5 hr. All transmission readings were converted to extinctions and the  $\Delta E$  values (initial extinction minus extinction at time  $t$ ) or, better, the corresponding percentage hemolysis values were plotted against time for a given concentration of a substance. Fig. 3 illustrates the type of curves obtained with various concentrations of gramicidin and methylol gramicidin. Complete hemolysis corresponds to a  $\Delta E$  of about 0.70–0.75.

Simultaneously with the testing of each new compound, hemolysis curves for gramicidin and methylol gramicidin were determined. An experiment was considered satisfactory only if the hemolysis curves for the two standard substances agreed closely with earlier curves obtained for these compounds. To ensure reproducibility it was necessary to keep the room temperature between 21° and 24°C. Higher temperatures caused a considerable increase in the rate of hemolysis. Only in a very few instances was it necessary to discard blood samples as unsuitable because of a lowered resistance toward gramicidin. A red cell suspension was considered unsuitable if the presence of 0.060 mg. gramicidin/100 ml. resulted in 40 min. in considerably more than 30% hemolysis.

Dimick (18) found a close correlation between the amount of tyrothricin added to a suspension of rat erythrocytes and the  $\Delta E$  values obtained after 2 min. and suggested the utilization of this linear relationship for the quantitative determination of tyrothricin. With gramicidin, however, no linear relationship between concentration and  $\Delta E$  was found in this laboratory. It would have been impractical, therefore, to express the hemolytic activity of a given concentration of a new gramicidin derivative as being equivalent to that of a definite concentration of gramicidin. A simpler method was chosen, namely, the determination of the *per cent* hemolysis produced in 2 hr. by that concentration of the new compound which was required for 50% inhibition of the growth of *Staph. aureus*. The "isobacteriostatic" concentration of gramicidin against *Staph. aureus*—0.045 mg./100 ml.—produced 100% hemolysis during this period of time under the experimental conditions described above.

### C. Results with Methylol Gramicidin

The results of a comparison between gramicidin and its reaction product with formaldehyde are summarized in Table I. The short incubation period of 4 hr. used by Lewis *et al.* (15) did not reveal any

TABLE I

*Bacteriostatic and Hemolytic Properties of Gramicidin and Methylol Gramicidin*

Substance	Mg./100 ml. for 50% inhibition		Per cent hemolysis by C50% S.A. in 2 hr.
	<i>Staph. aureus</i>	<i>Strep. Lancefield</i>	
Gramicidin	0.045	0.003	100
Methylol Gramicidin	0.023	0.006	15

difference in bacteriostatic activity between the two compounds. In the 18.5 hr. test used here, methylol gramicidin was found to be about twice as active against *Staph. aureus* and  $\frac{1}{2}$  as active against *Strep. Lancefield* as is gramicidin (see Figs. 1 and 2). There is, therefore, a definite difference between the antibacterial spectra of these two substances.

The hemolytic activity of methylol gramicidin in the 2 hr. test as described above was found to be about 15% of that of gramicidin, with individual values on 10 different blood samples ranging from 12 to 18%. Recent reports from the Western Regional Laboratory gave the hemolytic activity of methylol gramicidin as 20% (19) and 13% (20) of that of gramicidin. Lewis *et al.* (15) had stated earlier that concentrations of 0.005 mg. gramicidin/100 ml. and 0.06 mg. methylol gramicidin/100 ml. achieved 50% hemolysis of rat erythrocytes in 40 min. at room temperature. In a typical experiment with human red cells these concentrations were found to produce in 40 min. only 11 and 8% hemolysis, respectively, and even a 12-fold increase in gramicidin concentration to 0.06 mg./100 ml. produced only 21% hemolysis, as illustrated in Fig. 3.

#### *D. Preparation of Gramicidin Derivatives*

The chemical reactions with gramicidin were carried out generally in nonaqueous solvents, such as acetone, alcohol and glacial acetic acid. The reaction products were recovered, unless stated otherwise, by flocculation with 5–10 volumes of 0.1 N NaCl solution. The resulting precipitates were separated by centrifugation, washed with dilute NaCl solutions and water and dried *in vacuo* at room temperature over  $\text{CaCl}_2$ .

1. *Potassium Nitrite*. R-5: The addition of 0.066 millimol  $\text{KNO}_2$  in 1 ml. water to a solution of 0.01 millimol (28 mg.) of gramicidin in 5 ml.  $\text{CH}_3\text{COOH}$  at  $15^\circ\text{C}$ . produced a green color followed by turbidity. The mixture was left at room temperature for 45 min. and the reaction product was then flocculated. The yield was 14.3 mg. of a yellow powder. The hemolytic activity of R-5 (0.06 mg./100 ml.) was 47% of that of gramicidin; no effect on the growth of *Staph. aureus* was observed in concentrations up to 0.45 mg./100 ml.

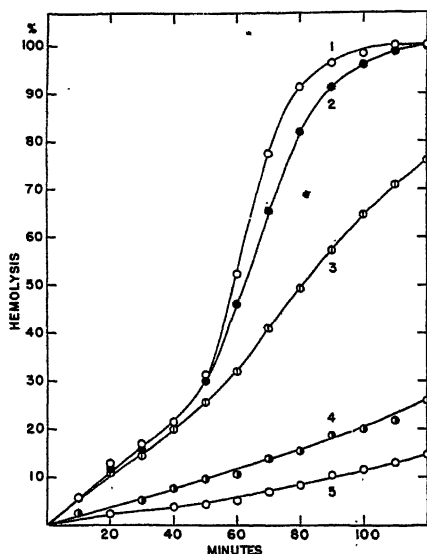


FIG. 3. Hemolysis of washed suspensions of human erythrocytes by gramicidin and methylol gramicidin.

The gramicidin concentrations in mg./100 ml. are Curve 1, 0.060; Curve 2, 0.045; Curve 3, 0.020; the methylol gramicidin concentrations are Curve 4, 0.060; Curve 5, 0.020 mg./100 ml.

The treatment of 28 mg. gramicidin in 5 ml.  $\text{CH}_3\text{COOH}$  in the same manner in absence of  $\text{KNO}_2$  led to the recovery of 24.5 mg. gramicidin, unchanged, as revealed by hemolytic and bacteriostatic tests. When acetone instead of glacial acetic acid was used as a solvent for gramicidin and  $\text{KNO}_2$  was added immediately after it had been dissolved in 0.06  $N$   $\text{HCl}$ , no reaction took place and 25 mg. gramicidin was recovered.

2. *Chromic Acid*. R-29: The addition of 9.3 mg. (0.093 millimol)  $\text{CrO}_3$  to a solution of 28 mg. gramicidin in 5 ml.  $\text{CH}_3\text{COOH}$  led to the rapid development of a dark color and the formation of a precipitate. After a reaction time of 2-3 min. the material was flocculated by  $\text{NaCl}$  and 21.2 mg. of a greyish substance was isolated. The reaction product inhibited the growth of *Staph. aureus* 50% in a concentration of 0.125 mg./

100 ml. (see Fig. 1) and the hemolytic activity at this concentration was 45% of that of an isobacteriostatic solution of gramicidin (see Fig. 4).

3. *Bromine*. R-21: To a solution of 28 mg. gramicidin in 5 ml. 95% alcohol was added 0.01 ml. bromine. The mixture was kept at 21–23°C. for 2 days and worked up in the usual manner. There was obtained 19.8 mg. of a substance, which had no effect on the growth of *Staph. aureus* in concentrations up to 0.58 mg./100 ml. and no hemolytic activity in a concentration of 0.20 mg./100 ml.

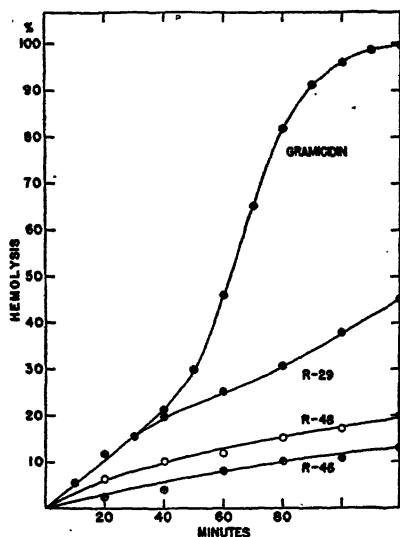


FIG. 4. Hemolysis of washed suspensions of human erythrocytes by gramicidin and three gramicidin derivatives.

The concentrations of the various compounds are those required for 50% inhibition of the growth of *Staph. aureus* at 18.5 hr. The hemolysis curve for an isobacteriostatic concentration of methylol gramicidin has been omitted as it practically coincides with the curve for R-46.

R-22: The experiment was repeated as above, except that  $\text{CH}_3\text{COOH}$  instead of alcohol was used as a solvent for gramicidin. The addition of bromine led immediately to the formation of a yellow precipitate. After a few minutes,  $\text{NaCl}$  solution was added and the reaction product was recovered as usual. There was obtained 38.6 mg. of a yellow powder, which had no effect on the growth of *Staph. aureus* in concentrations up to 0.50 mg./100 ml. A concentration of 0.20 mg./100 ml. showed 10% of the hemolytic activity of gramicidin (0.045 mg./100 ml.).

4. *Iodine*. R-17: 54.5 mg. iodine (0.215 millimol) was added to a solution of 28 mg. gramicidin in 5 ml. 95% alcohol. After the addition of one drop phosphate buffer ( $M/15$ , pH 6.5) the mixture was allowed to stand for 4 days at 21–23°C. There was

no apparent change in color during that time. The addition of 50 ml. 0.1 *N* NaCl solution precipitated brown material which was rendered colorless by the dropwise addition of 0.1 *N* Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> solution. The precipitate was then isolated, washed and dried in the usual manner. The yield was 23.4 mg. The concentration required for 50% inhibition of *Staph. aureus* was 0.097 mg./100 ml., and the hemolytic activity at this concentration was 11% of that of gramicidin.

R-43: A second preparation was carried out as above, but stood 5 days before being worked up. The yield was 22.9 mg. The concentration required for 50% inhibition of *Staph. aureus* was 0.107 mg./100 ml., for *Strep. Lancefield* 0.093 mg./100 ml. The hemolytic activity was 14% of that of gramicidin.

When twice as much (R-44) or three times as much (R-45) iodine was used under otherwise identical conditions, products resulted which did not inhibit the growth of either test organism in concentrations up to 0.30 mg./100 ml. At this concentration the compounds had 7-8% of the hemolytic activity of gramicidin.

R-48: In this large scale preparation, 1.40 g. gramicidin (0.50 millimol) was dissolved in 250 ml. alcohol and 2.00 g. iodine (7.9 millimols) was added, followed by 3 ml. phosphate buffer (*M*/15, pH 6.5). After 4 days standing at 21-23°C. the mixture was worked up as described for R-17. The yield was 958 mg. Fifty per cent inhibition of the growth of *Staph. aureus* was achieved at a concentration of 0.080 mg./100 ml. (see Fig. 1). At this concentration the material showed 20% of the hemolytic activity of gramicidin (see Fig. 4). The growth of *Strep. Lancefield* was inhibited 50% at a concentration of 0.055 mg./100 ml.

5. *Hydrochloric Acid*. R-18: To a solution of 50 mg. gramicidin in 2.5 ml. CH<sub>3</sub>COOH was added 2.5 ml. 10 *N* HCl. The mixture was kept at 37°C. for 24 hr. in a glass stoppered container and gradually turned violet-blue during that time. One-half of the solution was then evaporated to dryness *in vacuo* at temperatures below 35°C. and remaining traces of CH<sub>3</sub>COOH were removed by storage *in vacuo* over CaCl<sub>2</sub> and KOH. A purple powder was obtained in a yield of 18.3 mg. (R-18-A). The remaining half of the reaction mixture was poured into 15 ml. 0.1 *N* NaCl solution and worked up in the usual way. This resulted in a yield of 12.9 mg. of a purple powder (R-18-B). The bacteriostatic and hemolytic activities of these preparations are listed in Table II.

R-37: Using the same quantities as described above, four identical mixtures of gramicidin, CH<sub>3</sub>COOH and HCl were prepared. Three of these solutions were sealed immediately into Pyrex test tubes after the air had been replaced by nitrogen. The fourth tube was left open and all tubes were placed in an incubator at 37°C. The contents of the sealed tubes remained colorless throughout the incubation time. After various periods of time, the reaction products were flocculated with NaCl solution, washed and dried *in vacuo*. The results are summarized in Table II.

R-47: This preparation was a large scale duplicate of R-37-4. A solution of 1.40 g. gramicidin in 70 ml. CH<sub>3</sub>COOH was mixed with 70 ml. 10 *N* HCl in a 200 ml. Erlenmeyer flask and loosely stoppered. The mixture was incubated for 24 hr. at 37°C. The clear, dark blue-violet solution was then poured into 1,000 ml. 0.1 *N* NaCl solution and the resulting purple precipitate recovered, washed and dried as usual. The yield was 0.62 g. The concentration required for 50% inhibition of *Staph. aureus* was



TABLE II

*Bacteriostatic and Hemolytic Properties of Substances Obtained  
by the Treatment of Gramicidin with Hydrochloric Acid*

Experiment No.	Reaction time hr.	Mg./100 ml. for 50% inhibition <i>Staph. aureus</i>	Per cent hemolysis by C50% S.A. in 2 hr.
A. Sealed Tubes			
37-1	8	0.16	46
37-2	17	0.29	10
37-3	24	inactive*	3*
B. In Presence of Air			
37-4	24	0.33	2.7
18-A	24	0.23	10
18-B	24	0.18	8
47	24	0.21	5

\* In a concentration of 0.41 mg./100 ml.

0.21 mg./100 ml.; that for *Strep. Lancefield* was 0.44 mg./100 ml. The hemolytic activity was 5% of that of gramicidin.

6. *Hydroxylamine*. To 5 ml. portions of alcoholic solutions of gramicidin, each containing 28 mg. of this substance, were added 2.0-2.4 ml. portions of alkaline solutions of hydroxylamine. The mixtures were kept at 65-70°C. for 17-18 hr. and the reaction products then were isolated in the usual manner. The yields were 22-25 mg. Variation of the amounts of  $\text{NH}_2\text{OH}$  between 1.2 and 2.4 millimols and that of NaOH between 0.16 and 0.26 millimol had little effect on the properties of the reaction products. These substances inhibited *Staph. aureus* 50% at concentrations between 0.05 and 0.07 mg./100 ml. and had hemolytic activities close to that of methylol gramicidin.

R-46: To 1.40 g. gramicidin (0.50 millimol) in 250 ml. alcohol was added 100 ml. of a solution containing 60 millimols  $\text{NH}_2\text{OH}$  in 0.12 N NaOH. The mixture was kept for 17 hr. at 68-72°C. (reflux condenser), and the reaction product was then flocculated with 1,000 ml. 0.1 N NaCl solution and isolated in the usual manner. The crude product (1.2 g.) contained some alcohol-insoluble material. Extraction with two 20 ml. portions of alcohol and evaporation at room temperature yielded 0.67 g. of an alcohol-soluble substance. The concentration of this material required for 50% inhibition of *Staph. aureus* was 0.077 mg./100 ml. (see Fig. 1), and the hemolytic activity at this concentration was 13% of that of gramicidin, as shown in Fig. 4. The growth of *Strep. Lancefield* was inhibited 50% at a concentration of 0.093 mg./100 ml.

7. *Sodium Hydroxide*. The reaction between gramicidin and formaldehyde was carried out in alkaline solution (15), and the treatment with hydroxylamine, de-

scribed above, was also performed in presence of NaOH. It seemed advisable, therefore, to test whether sodium hydroxide itself would have a modifying influence on gramicidin.

R-32: The conditions used in this experiment were identical with those described by Lewis *et al.* (15), except that water was substituted for formaldehyde. To a solution of 0.25 g. gramicidin in 5 ml. alcohol was added 3 ml. 0.18 *N* NaOH, and the mixture was kept at 50°C. for 48 hr. Flocculation with NaCl solution and washing then was carried out as described (15), and 0.20 g. reaction product was isolated. This material inhibited the growth of *Staph. aureus* 50% at a concentration of 0.10 mg./100 ml. and had 46% of the hemolytic activity of an isobacteriostatic gramicidin solution.

In a series of 23 experiments, 5 volumes of a 0.002 *M* solution of gramicidin in alcohol (0.56 g./100 ml.) were mixed with 2 volumes of aqueous NaOH solution, ranging in strength from 0.05 to 0.375 *N* and kept at 70°C. for 17 hr. The reaction products were isolated in the usual manner. Sodium hydroxide concentrations up to 0.1 *N* produced gramicidin derivatives which had lost more of the antibacterial effectiveness than of the hemolytic activity of the starting material. A further slight increase in NaOH concentration (0.12–0.13 *N*) yielded derivatives which had about the same antibacterial activity as the first group, but showed a marked reduction in hemolytic activity. Finally, when the upper limits of the NaOH concentrations used here were approached, a destruction of antibacterial properties took place again. These findings are summarized in Table III. Heating in sealed

TABLE III

*Bacteriostatic and Hemolytic Properties of Substances Obtained by the Treatment of Gramicidin with Sodium Hydroxide at 70°C. for 17 Hr.*

Experiment No.	Normality of NaOH	Mg./100 ml. for 50% inhibition <i>Staph. aureus</i>	Per cent hemolysis by C <sub>50</sub> % S.A. in 2 hr.
311	0.05	0.08	87
312	0.10	0.12	45
38,41,42	0.12–0.13	0.08–0.13	11–17
331,39,40	0.25–0.38	0.25 and more	8–16
28	0.13*	0.31	31
332	0.25**	0.19	2

\* Acetone instead of alcohol used as solvent.

\*\* Sealed tube, nitrogen atmosphere.

tubes after air had been replaced by nitrogen usually resulted in a marked decrease in hemolytic activity as compared to the parallel experiment exposed to air. The use of acetone as solvent for gramicidin instead of alcohol protected hemolytic properties and led to a considerably greater loss in antibacterial activity than did the same experiment in alcoholic solution (see Table III). As an example for this series of experiments, the preparation of R-42 is described: 1.40 g. gramicidin was dissolved in 250 ml. alcohol and 100 ml. 0.121 *N* NaOH was added. The mixture was kept at 70°C. for 17 hr. (reflux condenser) and showed a light yellow color and a slight precipitate. Flocculation of the reaction product was carried out as usual, and 1.28 g. product was obtained. This material was extracted twice at room temperature with 20 ml. portions of alcohol. From the combined extracts 0.70 g. alcohol-soluble material was recovered, and there remained an alcohol-insoluble residue of 0.56 g. The concentration of the alcohol-soluble material required for 50% inhibition of *Staph. aureus* was 0.077 mg./100 ml.; that for *Strep. Lancefield* was 0.067 mg./100 ml. The hemolytic activity was 17%.

## DISCUSSION

Gramicidin reacts with remarkable ease with a variety of reagents. No definite statement can be made so far about the structural changes which occur in these reactions, but it is most likely that substitutions in the tryptophan part of the molecule and hydrolytic cleavage of peptide bonds take place. Fraenkel-Conrat and coworkers (21) have supplied evidence to show that formaldehyde is added to the  $\alpha$ -carbon atom of the indole ring in the tryptophan groups of gramicidin to form methylol gramicidin. The changes in properties which were observed in our experiments under identical conditions, but in absence of formaldehyde (R-32), suggest that methylol gramicidin differs from the parent compound structurally by more than the simple replacement of several hydrogen atoms by  $\text{CH}_2\text{OH}$  groups.

A comparison of the various gramicidin derivatives shows that the bacteriostatic and hemolytic properties of gramicidin can be changed independently of each other, which leads to the conclusion that these two activities are associated with different parts of the gramicidin molecule. Nitrite, for example, yielded a product (R-5) with about half the hemolytic activity of gramicidin, but the new substance had no

effect on the growth of *Staph. aureus* in a concentration 10 times as high as the gramicidin concentration required for 50% inhibition. Hydroxylamine, by contrast, gave a product (R-46) which had retained 58% of the effectiveness of gramicidin against *Staph. aureus*, but showed at the isobacteriostatic concentration only 13% of the hemolytic activity of the parent compound.

The change in antibacterial spectrum as a result of the chemical treatment of gramicidin is characterized by a considerable reduction in the effectiveness of the new compounds against *Strep. Lancefield*, as compared to gramicidin and methylol gramicidin. It remains to be seen whether this loss in activity is compensated for by an increased effectiveness against other organisms.

The determination of the 50% inhibition values rather than the concentration required for complete inhibition of visible bacterial growth was chosen, because the 50% values depend on the evaluation of the turbidity in a number of tubes and not on the appearance of a single tube. The fluctuations in the numerical results from one experiment to the next are, therefore, smaller than in the alternative method. The selection of the first tube without visible growth is particularly difficult with *Strep. Lancefield*. With this organism, a slight turbidity was still visible with gramicidin concentrations as high as 0.10 mg./100 ml., which is 30 times the concentration required for 50% inhibition. Subcultures from such tubes showed complete growth within 24 hr. and required, when diluted  $10^{-3}$  with sterile broth and tested again, 0.007 mg. gramicidin/100 ml. for 50% inhibition. With *Staph. aureus* as test organism visible growth ceased at gramicidin levels of 0.08–0.10 mg. gramicidin/100 ml.

It was important in all tests for antibacterial activity to keep incubation time and temperature constant. In a typical experiment with *Strep. Lancefield*, for example, the gramicidin concentration required for 50% inhibition was 0.0034 mg./100 ml. after 18.5 hr. incubation. This value increased to 0.0045 at 22 hr., 0.0052 at 26 hr. and 0.0059 mg./100 ml. at 48 hr. With *Staph. aureus* corresponding values were 0.045 mg./100 ml. at 18.5 hr., 0.062 at 22 hr., and after 48 hr. incubation even tubes containing 0.20 mg./100 ml. showed 60% growth. Similar observations were made with the new gramicidin derivatives, which proves that their action is bacteriostatic rather than bactericidal.

## •ACKNOWLEDGMENTS

The work described in this report has been supported by a grant from the Wallerstein Company, Inc., New York. The authors are indebted to Mr. Leo Wallerstein for a generous gift of gramicidin and methylol gramicidin.

## SUMMARY

1. Treatment of gramicidin with nitrite, chromic acid, bromine, iodine, hydroxylamine, hydrochloric acid and sodium hydroxide produced derivatives which differed considerably from the starting material in their toxicity and in their bacteriostatic and hemolytic activities.

2. The activity of the new compounds against *Staph. aureus* varied in the range from 14 to 58% of that of gramicidin, with the exception of the reaction products obtained with nitrite and bromine, which were inactive at the levels tested.

3. The hemolytic activity of isobacteriostatic concentrations of the gramicidin derivatives varied from 2 to 87% of that of gramicidin.

4. Methylol gramicidin as well as the new derivatives differ in their antibacterial spectra from the parent compound.

5. The conditions used for the preparation of methylol gramicidin produced a new gramicidin derivative even in absence of formaldehyde. This finding suggests that methylol gramicidin differs from the starting material structurally by more than the replacement of several hydrogen atoms by methylol groups.

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# The Acid Dissociation of Acetyl and Butyryl Phosphoric Acids

Fritz Lipmann and L. Constance Tuttle

*From the Biochemical Research Laboratory, Massachusetts General Hospital, Boston 14, Mass.*

Received March 7, 1947

Early during the work with acetyl phosphate (1) we became aware that its solutions exhibited a surprisingly acid buffer optimum. This indicated a rather profound change in the acid dissociation of the phosphoric acid group. Titration experiments are reported here which show that in acetyl phosphate the dissociation of the second hydroxyl group ( $pK'$  4.9) approaches very closely that of the carboxyl in acetic acid ( $pK'$  4.65).

The spontaneous hydrolysis of acetyl phosphate precludes too great accuracy of titration experiments. Spontaneous hydrolysis is small enough, however, at room temperature to make the reported experiments reasonably reliable. Decomposition was checked at the end of every experiment and amounted to not more than 3–7%. Most of this occurs in the second period of titration, *i.e.*, in the range of low pH. The accuracy is greatest, therefore, in the region of the second dissociation; the stability maximum of acetyl phosphate is between pH 5 and 6 (1).

The instrument used was a pH meter of the Gamma Instrument Company, New York; it is similar to the Beckman instrument. A glass electrode of the Beckman type was used as obtained from the same company. The measurements were made against a calomel electrode in saturated potassium chloride.

Crystalline disilver acyl phosphates were used. The preparation of these compounds was described in an earlier publication (1). For the titration the silver salt was converted to the sodium salt. A nearly 0.1 *M* solution of disodium acetyl phosphate was prepared by homogenizing appropriate amounts of silver salt in the cold with 10% excess of sodium chloride solution. The silver chloride was removed by filtration and the solution analyzed. Conveniently small portions of this solution were stored without loss in a deep freeze box at  $-35^{\circ}\text{C}$ . and used for individual experiments.

For the titration, exactly 5 millimoles of this solution were measured into a Pyrex beaker of 25–30 ml. capacity and filled with water to 10.0 ml. initial volume. The



actual titration was done with 0.2 *N* hydrochloric acid, added from a burette that dipped into the solution. The beaker stood in a larger container of water at room temperature. Titration of the sodium salt with acid was preferred because the compounds are considerably more stable at a neutral or slightly acid reaction than in a strongly acid solution.

The results are summarized in Table I and in Figs. 1 and 2. Examples of titration curves are represented in Fig. 1. In Table I the dissociation constants are listed as obtained from the best experiments, either graphically or by calculation according to Van Slyke (2). The most

TABLE I  
*Apparent Dissociation Constants of Acetyl and Butyryl Phosphoric Acids*

Acid	$\text{pk}_1'$	$\text{pk}_2'$	Temperature
Monoacetyl phosphoric	1.17	4.80	°C.
		4.86	22
		4.96	20.8
		5.08	21.5
Monobutyryl phosphoric	1.17	5.10	24.0

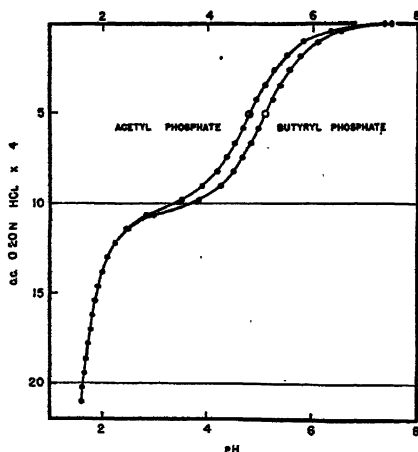


FIG. 1. Electrotitration Curves. Exactly 10 ml. of 0.05 molar sodium acyl phosphate were titrated with 0.2 *N* hydrochloric acid. The normality of the titrating acid was thus four times the molarity of the titrated compound. To facilitate comparison the actual amounts of added acid appear in the table multiplied by the factor four.

numerous titrations were carried out with acetyl phosphate. Even when choosing our best experiments a relatively large variation remains. This is ascribed to the fragility of the compound. The observed decrease from acetyl to butyryl derivative, however, seems real. Recent experiments by Lehninger (3) with octoyl and palmityl phosphate bear out the acid weakening effect of the lengthening of the acyl carbon chain. For octanoyl and palmityl phosphoric acid he obtained  $\text{pk}_2'$  values of 5.4 and 6.2 respectively. Preliminary titration of propionyl, using a not completely pure preparation, yielded  $\text{pk}_2'$  values of 4.95 and 5.09 at 25°C.

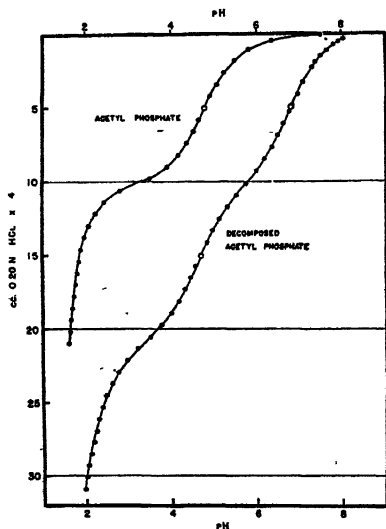


Fig. 2. Comparison of Electrode Titration Curves of Acetyl Phosphate Before and After Hydrolysis.

Because of the greater instability at strongly acid reaction, the data for  $\text{pk}_1$  are less reliable. The values indicate that the acid strength of the first hydroxyl is relatively less affected by anhydridization with fatty acid.

In Fig. 2 titration curves are compared as obtained before and after hydrolysis of acetyl phosphate. For decomposition, part of the solution was heated with excess alkali and brought back to phenolphthalein red with hydrochloric acid. The curve for decomposed acetyl phosphate

agreed rather exactly with a curve for an equimolar mixture of acetate and phosphate.

In Table II a survey is given of the acid strength of various derivatives of phosphoric acid. With substitution, a strengthening invariably occurs which, in acetyl or creatine phosphate, may amount to as much as 2 pk units. The theoretical aspects of this acid strengthening in substituted phosphoric acids have been recently discussed by Kumler and Eiler (8).

TABLE II  
*Comparison of the Acid Dissociation of Various Phosphoric Acid Derivatives*

Acid	pk <sub>1</sub> '	pk <sub>1</sub> '	Reference
Orthophosphoric	1.97	6.83	Van Slyke (2)
α-Glycerol phosphoric	1.40	6.44	Kiessling (4)
Fructose-6-phosphoric (Neuberg ester)	0.97	6.11	Meyerhof and Lohmann (5)
Choline phosphoric		5.62	Zamecnik, Brewster and Lipmann (6)
Octanoyl phosphoric		5.4	Lehninger (3)
Acetyl phosphoric	1.2	4.9	this paper
Creatine phosphoric		4.58	Meyerhof and Lohmann (7)

#### ADDENDUM

##### *Preparation of Lithium Acetyl Phosphate*

Recently we learned how to prepare lithium acetyl phosphate and used this compound in some tests. Lithium acetyl phosphate is readily soluble in water and may be used conveniently in physiological experiments. Therefore, a short description of its preparation will be given here.

*Preparation of lithium acetyl phosphate.*—In the manner earlier described in detail (1), 32 g. silver phosphate (Merck) and 11 ml. of 90% phosphoric acid (Mallinkrodt) are mixed and suspended in 20 ml. ether, and 17 ml. of acetyl chloride, in an equal ether volume, are added. The well-cooled reaction mixture is now treated with 80 ml. of ice water, followed carefully by 85 ml. of 4 *N* lithium hydroxide in portions. The solution is brought to pH 3.5 to 4. The silver chloride is removed by filtration and the fluid extracted twice with three times its volume of ether. This treatment removes free acetic acid. The aqueous solution, still kept cold, is now further neutralized with 50–60 ml. of

4 *N* lithium hydroxide to pH 8. The bulky precipitate of lithium phosphate is removed by filtration. The filtrate is free of inorganic phosphate. To 160 ml. are added slowly, in about five portions, 1000 ml. of ethyl alcohol. Generally a crystalline white precipitate is formed; with somewhat hasty alcohol addition this may be more or less gelatinous. The lithium acetyl phosphate is collected on a Buchner funnel and washed there with alcohol and ether. It is finally dried over phosphorus pentoxide in a vacuum desiccator. If carefully prepared, the analysis corresponded with dilithium acetyl phosphate, without reprecipitation. The yield was 7-8 g.

$C_2H_3O_5PLi$	Calculated.	Acetic acid 39.5, P 20.4
151.8	Found	Acetic acid 39.7, P 20.1

### SUMMARY

The apparent dissociation constants of acetyl and butyryl phosphoric acids were determined by electrotitration. The obtained values were:  $pk_1'$  of 1.17 and  $pk_2'$  of 4.87 for acetyl, and  $pk_1'$  1.17 and  $pk_2'$  5.1 for butyryl phosphoric acid.

The preparation of lithium acetyl phosphate is described.

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# The Metabolism of *meso*-Inositol in the Rat \*

V. D. Wiebelhaus, Joseph J. Bethel and Henry A. Lardy

*From the Department of Biochemistry, College of Agriculture,  
University of Wisconsin, Madison*

Received March 10, 1947

The almost ubiquitous occurrence of *meso*-inositol in plant and animal tissues, together with its structural relation to the 6-carbon sugars, (1) has prompted investigation and stimulated speculation upon its biological functions.

Inositol is required for the growth of certain yeasts (2, 3), a mutant strain of *neurospora* (4), and for animals under some conditions (5, 6). The vitamin-like properties of inositol have been reviewed by Woolley (7).

Because of its chemical constitution inositol has long been considered a likely intermediate in the natural synthesis of aromatic compounds from carbohydrates. Fischer (1) has recently discussed the possibility of inositol being a "reserve" carbohydrate in living cells and suggested a possible role for inositol in the interconversion of various hexoses.

Since the first work of Vohl (8) in 1858, numerous investigations have been made of the metabolism of inositol by animals. Vohl, and later Needham (9), reported finding large amounts of inositol in the urine of human subjects. Needham also reported a vigorous excretion of inositol over long periods of time by polyuric rats on an inositol-free diet (10). These and other early studies of the metabolism of inositol by animals were hampered by lack of suitable quantitative methods for accurately determining this compound in biological material. No adequate proof has yet been offered that animal tissues can synthesize inositol, for Woolley (5) and Handler (11) have presented evidence that intraintestinal synthesis may furnish inositol to the rat. Furthermore, Woolley has shown (12) that the seeming synthesis of inositol by the chick embryo (Needham (13)) is actually a liberation of bound inositol from the yolk constituents.

The data on the absorption of inositol vary widely. Starkenstein (14) found only slight absorption by rabbits of injected or fed inositol. Mayer (15) reported that, when given orally to rabbits, 2.0–2.4% of the inositol appears in the urine but when given subcutaneously 26–52% of the dose is thus excreted. Anderson and Bosworth (16) found that when human subjects were fed inositol about 9% appeared in the

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\* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the University Research Committee.

urine and none was found in the feces. Working with dogs, Anderson (17) found inositol to be slowly absorbed and only a small portion of the material fed was excreted by the kidneys. He found no rise in the respiratory quotient.

The possible utilization of inositol as a carbohydrate has been investigated by many workers, mainly with inconclusive results. Das and Guha (18) reported increased oxygen consumption by brain, kidney and liver tissue of white rats when inositol was added. Young (19) could not confirm these results. Kulz (20) could find no increase in urinary sugar excretion of diabetic or phlorizinized animals after the administration of inositol. Von Mering (21) and Mayer (15) were unable to detect increased liver glycogen as a result of inositol administration.

Greenwald and Weiss (22) administered inositol orally to phlorizinized dogs over comparatively extended periods of time and found a slight but unmistakable increase in the urinary glucose:nitrogen ratio. They concluded that, in the dog, the inositol was slowly and incompletely converted to glucose.

Proof that at least some glucose is formed from inositol by the rat was presented by Stetten and Stetten (23). The injection of inositol containing stably bound deuterium into a phlorizinized rat resulted in the excretion of urinary glucose containing sufficient deuterium to indicate a minimum of 7% conversion of the injected inositol to glucose.

The experiments reported here were designed to determine the rate of absorption of inositol, its possible effect on liver glycogen, and the influence of inositol on starvation ketosis in the rat. Some determinations of the inositol content of certain rat tissues are also presented.

## EXPERIMENTAL AND RESULTS

Quantitative determinations of inositol were made by the yeast (*S. carlsbergensis*) growth assay of Atkin *et al.* (24). Growth was measured by turbidity as determined with a photoelectric colorimeter using a filter with maximum transmission at 660 m $\mu$ . The assay range extended from 1 to 15% per tube.

### *Absorption of Inositol*

For the study of absorption the procedure of Cori (25) was followed. A series of white rats, all approximately 200 g. in weight, was fasted 24 hours. The animals were then given an aqueous solution of the test material by means of a small rubber catheter employed as a stomach tube. Dosages were proportional to (Weight)<sup>.73</sup>, a factor which Brody (26) has shown to parallel the "metabolically effective" weight of animals of all sizes more closely than do surface area formulae. For a 185 g. rat the dose amounted to 258 mg. of inositol. A calibrated hypodermic syringe was used to measure the solution; the test solution being followed by a minimum volume of distilled water to rinse the catheter. Controls were given equal volumes of distilled water by the same method and all animals were then placed in metabolism cages.

At various time intervals the animals were sacrificed. The upper and lower extremities of the gastrointestinal tract were ligated, the tract

removed and quickly rinsed with distilled water. The contents of the tract were then washed into volumetric flasks with hot distilled water and, when cool, diluted to a definite volume. Urine specimens were collected from the time of dosage to the time of sacrifice. Aliquots of each sample were hydrolyzed by refluxing with 20% HCl for six hours to liberate all bound inositol for purposes of microbiological assay (27, 28). The hydrolyzates were evaporated to dryness under reduced pressure, taken up in water, adjusted to pH 4-5 and made up to a definite volume. Inositol was determined in both hydrolyzed and unhydrolyzed samples.

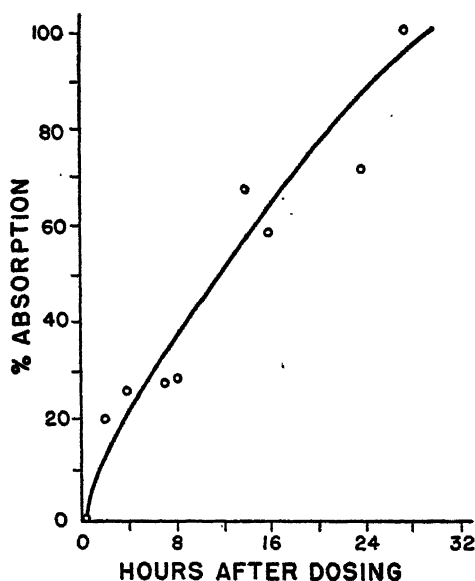


FIG. 1. Rate of inositol absorption in fasted rats.

The intestinal contents of several fasted rats were found to contain from 8 to 12 mg. of total inositol and this amount was therefore subtracted from all experimental values in calculating the amount of inositol which had been absorbed. The data obtained are summarized in Fig. 1. The rate of absorption was found to be comparatively slow, but appears to be complete at about 28 hours.

A comparison of the assay values for the hydrolyzed and unhydrolyzed samples indicated that no conversion of administered inositol to



a bound form had occurred in the intestine. However, about one-half of the 8–12 mg. of inositol present in the tract of fasted animals (or of the experimental animals after absorption of the test dose was complete) was in a form not available to the strain of yeast used in these assays.

The urinary excretion of inositol varied from 3 to 5 mg./rat for a 24 hour period and was not significantly greater for the rats which had received from 260 to 335 mg. of inositol *per os* than for the fasted control rats. The limit of accuracy of the inositol assay permits the conclusion that urinary excretion accounts for less than 1% of the administered inositol.

The absorption of inositol monophosphate was also studied. From 150 to 200 mg. of the monophosphate isolated from soybean lipositol (27) was administered to each of eight 200 g. rats. The rate of absorption appeared to be more rapid than for inositol, especially during the first 2 hours. Approximately 80% of the dose had been absorbed in from 1 to 2 hours and complete absorption had occurred in rats killed at 12 and 24 hours. In all animals killed later than 2 hours after administering inositol monophosphate, almost all of the inositol in the intestinal contents was found to be in a form available to the assay organism. Since the original isolated inositol monophosphate gave a growth response equivalent to only 8–15% of its contained inositol, it appeared that the phosphoric ester was rapidly hydrolyzed in the intestine. The initial rapid disappearance of inositol might indicate an extremely rapid absorption of the inositol monophosphate *per se* with subsequent slow absorption of the free inositol which was presumably liberated by intestinal phosphatases.

Two of the rats which received inositol monophosphate excreted greater than normal amounts of inositol in their urine. The amounts were 10 mg. in 3 hours and 19 mg. in 8 hours, respectively. The other rats given inositol monophosphate excreted normal amounts of inositol and the differential assay showed no indication of bound forms of inositol in any of the urine specimens.

### *Tests for Glycogenesis*

Previously reported attempts to demonstrate increased liver glycogen were either negative or inconclusive. However, in none of these studies, including the recent work of Stetten and Stetten (23), were precautions taken to avoid glycogenolysis at death. Davenport and Davenport (29) and Cori (30) have shown that glycogen

breakdown is unavoidable unless the animal is first anesthetized and the tissue sample removed from the living animal. By employing this technique it seemed possible that any slight increment in liver glycogen resulting from inositol would be detectable.

White rats were fasted for 24 hours and then given aqueous solutions of either inositol or glucose by stomach tube. The dosages were again proportional to  $W^{.73}$  and for a 175 g. rat amounted to 244 mg. of the compound fed at the lower level and 590 mg. at the higher level. Control rats were given equal volumes of distilled water. At various time intervals the rats were anesthetized by intraperitoneal injections of amytal. The livers were removed rapidly and immediately frozen in a bath of dry ice and acetone. They were then crushed and digested in hot 30% potassium hydroxide. The glycogen was separated and hydrolyzed by the method of Good *et al.* (31). Reducing sugar values were determined by the copper-iodometric method of Shaffer and Somogyi (32). The results are summarized in Table I.

TABLE I  
*Liver Glycogen in Inositol-Fed Rats*

No. of rats*	Material fed	Dosage mg. $\times W^{.73}$	Hours from dosage to sacrifice	Per cent liver glycogen (based on wet weights of livers)
6	Water	—	4-6	.11 $\pm$ .05
10	Water	—	8-16	.08 $\pm$ .04
5	Water	—	19-28	.06 $\pm$ .03
3	Glucose	5.7	2-6	.51 $\pm$ .10
3	Glucose	5.7	12-16	.13 $\pm$ .05
1	Glucose	13.8	4	1.25
2	Glucose	13.8	8-12	.60 $\pm$ .20
2	Glucose	13.8	16-24	.09 $\pm$ .03
4	Inositol	5.7	4-8	.09 $\pm$ .03
5	Inositol	5.7	12-16	.11 $\pm$ .04
5	Inositol	5.7	19-28	.09 $\pm$ .02
2	Inositol	13.8	4-6	.07 $\pm$ .01
2	Inositol	13.8	8-16	.08 $\pm$ .02
3	Inositol	13.8	20-24	.07 $\pm$ .01
2	Inositol-monophosphate	5.7	2-4	.06 $\pm$ .03
1	Inositol-monophosphate	5.7	6	.16
1	Inositol-monophosphate	5.7	8	.08
1	Inositol-monophosphate	5.7	24	.13
1	Inosose	5.7	24	.07

\* All animals were subjected to a preliminary fasting period of 24 hours.

Increased glycogen was found in the livers of the animals given glucose but no such increase over the control was shown by the animals given inositol at two levels of dosage. In the few animals given inositol monophosphate the liver glycogen appears to be sustained at a slightly higher level during the 8-24 hour period than in the case of the controls, but the difference is very slight.

### *Anti-Ketogenic Activity of Inositol*

Since Stetten and Stetten (23) had found only a relatively minor conversion of administered inositol to glucose in their experiment and our absorption data indicated comparatively slow absorption of inositol it was concluded that a better measure (than glycogenesis) of the possible utilization of inositol as a carbohydrate would be its effect on ketosis.

A series of white rats (average weight about 180 g.) was fed the high fat-low protein diet of Tidwell and Treadwell (33) for 18 days. They were then fasted 48 hours and divided into four groups which received the following treatments respectively: I, 625 mg. of glucose by stomach tube; II, 625 mg. of inositol by the same route; III, 625 mg. of inositol injected intraperitoneally; IV, distilled water by stomach tube. The rats were kept in metabolism cages, given water *ad libitum* and the urines were collected during the following 24 hours. Total ketone bodies present in the urines were determined by the method of Tidwell and Treadwell (33).

The results are summarized in Table II. A marked antiketogenic effect was observed in the group which received glucose. Inositol given orally or intraperitoneally definitely alleviated the ketosis. The rats receiving glucose excreted less ketone bodies to the extent of 65 mg. less acetone than the controls, while those given inositol (Lots II and III) excreted an amount equivalent to 23 mg. less acetone. On this basis, inositol appeared to be approximately one-third as effective as glucose in alleviating ketosis in white rats.

As a check on the possibility that the differences in levels of the ketone bodies found in the urines might have been due to gluconeogenesis from body proteins, the individual urines were analyzed for total nitrogen by a semimicro Kjeldahl method as described by Umbreit and Bond (34). The data on urinary nitrogen, also given in Table II, show that the administration of either glucose or inositol

TABLE II  
*Effect of Inositol on Fasting Ketosis*

Group	No. of rats	Material given	Ketone bodies determined as acetone in 24 hr. urine	Total nitrogen in 24 hr. urine
I	4	625 mg. glucose—orally in 2.4 ml. solution	mg. 15*	mg. 52*
II	4	625 mg. inositol—orally in 2.4 ml. solution	56	58
III	4	625 mg. inositol—intraperitoneally in 2.4 ml. soln.	59	65
IV	4	2.4 ml. H <sub>2</sub> O	80	74

\* Each value represents the average daily excretion per rat in the respective group.

resulted in a decreased nitrogen excretion. The antiketogenic effects of these compounds were therefore not the result of gluconeogenesis from body protein.

#### *Distribution of Inositol in Rat Tissues*

Since conversion of inositol to liver glycogen could not be detected, it was decided to study the possible deposition of inositol as such in various tissues of the rat.

Six white rats, averaging 250 g. each, were divided into three groups. Group I was taken directly from a stock ration and sacrificed. Groups II and III were fasted 24 hours. Group II was then given water by means of a catheter and Group III was given an equal volume of an inositol solution. The latter two groups were sacrificed six hours later. Blood samples and the livers, hearts and testes were removed. The tissues were digested and the inositol freed by refluxing for six hours in 20% HCl (28). The inositol in each tissue was determined by the microbiological assay. The results are summarized in Table III. The administration of inositol caused no appreciable changes in the tissue content of this compound, except in the case of heart. The hearts of the two fasted rats were found to contain 95 and 97 mg.-% of inositol while those of the rats which had been fed inositol contained 123 and 152 mg.-%, respectively. This marked increase is particularly interesting since Woolley (7) and Winter (35) had found comparatively high levels of inositol in beef heart.

TABLE III

*Influence of Fasting and Inositol Feeding on the Inositol Content of Various Rat Tissues*

Group No.	Fasting period	Material fed	Hours from dosage to sacrifice	mg.-% inositol*			
				Liver	Testes	Blood	Heart
I	0	—	0	132	126	31	78
II	24	H <sub>2</sub> O	6	155	115	34	97
III	24	800 mg. inositol	6	166	110	38	137

\* Values given are the average for the 2 rats in each group.

## DISCUSSION

In spite of the precautions of removing the livers from amytilized animals and immediately freezing them, no significant increase in liver glycogen of fasted rats given inositol was obtained. Our experiments on absorption show that, at the level of dosage employed in the absorption and liver glycogen experiments, the inositol was completely absorbed only after 24-28 hours. Therefore, any glucose, or other similarly utilizable material, which may have been formed from the inositol may well have been metabolized in the fasted animals as rapidly as formed without causing any detectable deposition of liver glycogen. Although the utilization of inositol is evidently slow, it would appear that it is metabolized as carbohydrate by the rat, since its administration alleviates ketosis and at the same time reduces the amount of total nitrogen excreted in the urine. It is logical to suppose that an antiketogenic function may be observed if the material given is utilized as a carbohydrate in the fasted animal without a prior conversion to glycogen as such.

It is also of interest that there is a marked increase in the inositol content of the heart of the rat after inositol has been administered to the animal following a preliminary starving period of 24 hours.

## SUMMARY

1. When white rats of approximately 200 g. weight were given 250 mg. of inositol in aqueous solution, 24-28 hours were required for

complete absorption. Urinary excretion was found to account for less than 1% of the inositol given.

2. No increase in the liver glycogen of white rats was found after the administration of inositol.

3. Inositol, given orally or intraperitoneally, functioned anti-ketogenically in the white rat.

4. The fasting of rats for 24 hours did not influence the inositol content of their blood, liver, testis, or heart tissues. The oral administration of inositol to fasted rats resulted in an appreciable increase in the inositol content of the heart, but caused no appreciable change in the other tissues studied.

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# Composition of Buffalo Milk Fat

C. P. Anantakrishnan, V. R. Bhalerao and T. M. Paul

*From the Indian Dairy Research Institute, Bangalore, India*

Received March 10, 1947

## INTRODUCTION

A considerable amount of work has been carried out on the properties and composition of cow's milk fat, but comparatively little attention has been paid to the milk fat of other mammals. In India, the buffalo forms a very important source of milk and butter fat, yet, apart from the work of Bhattacharya and Hilditch (1), no information seems to be available on the detailed composition of buffalo butter fat. It is generally found that the analytical constants of cow milk fat are affected within a very short period by the fat supplemented to the animal along with the basal diet. Since buffalo's milk is noted for its high fat content, it is of interest to know the effects of feeding oils and fats on the composition of the milk fat of such high yielding animals.

This paper will present the data of a series of experiments carried out to study the influence of supplementing a basal ration of buffaloes with fats of different levels of unsaturation on the component acids of resulting butter fat.

## EXPERIMENTAL

Murrah buffaloes of almost the same age, body weight, stage of lactation and milk yield were selected for the experiment from the herd of the Indian Dairy Research Institute. They were divided into groups of three each. The normal concentrate ration of the Institute herd consisted of a mixture of wheat bran, grain, groundnut (peanut) cake and grain husk in the proportion of 4.0:2.0:1.5:2.5. This mixture was fed at the rate of 1 lb. for every 2 lbs. of milk produced by the animal. The roughage consisted of 70 lbs. of green grass (a mixture of Guinea and Napier grasses) and 3 lbs. of straw. The animals were kept on this ration for a week. After this control period, each animal was fed with a supplement of 0.5 lb. of fat on the first day. This was increased to 1 lb. on the second day and 1.5 lbs./day thereafter, for a fortnight. The control animals were given only the normal ration during the entire time.



The groups were fed as follows:

- I. Basal ration.
- II. Basal ration plus 1.5 lbs. of coconut oil (iodine value 8.2).
- III. Basal ration plus 1.5 lbs. of cottonseed oil (iodine value 108.6).
- IV. Basal ration plus 1.5 lbs. of groundnut oil (iodine value 89.2).
- V. Basal ration plus 1.5 lbs. of sesame oil (iodine value 109.4).
- VI. Basal ration plus 1.5 lbs. of hydrogenated coconut oil (i.v. 4.2).
- VII. Basal ration plus 1.5 lbs. of hydrogenated groundnut oil (i.v. 70.4).

The iodine value of all milk fats were observed at the end of every 5 days. The oil feeding was stopped after 15 days, when a day's milk was collected and the butter fat was recovered for detailed analysis. The general analytical characteristics of the milk fats of the buffaloes are presented in Table I.

TABLE I  
*Analytical Characteristics of Buffalo Milk Fat*

Group	Fat supplement	B.R.* reading	Iodine value	Reichert value	Polenske value	Krischner value	Saponifi- cation equivalent
I	Control	41.0	26.0	32.24	1.95	28.41	240.4
II	Coconut oil	42.7	28.6	30.08	1.85	18.43	242.2
III	Cottonseed oil	43.1	31.5	26.24	0.86	22.16	252.2
IV	Groundnut oil	43.3	33.1	23.33	1.18	19.30	251.3
V	Sesame oil	43.0	33.8	29.67	0.92	24.52	247.7
VI	Hydrogenated coconut oil	40.6	24.3	29.60	1.41	24.81	241.1
VII	Hydrogenated groundnut oil	42.5	34.2	25.50	0.83	21.89	252.3

\* Butyro-refractometer reading at 40°C.

The component fatty acids in the 7 milk fats were determined by subjecting them to detailed analysis by ester fractionation according to the method of Hilditch as modified by Smith and Dastur (6). The fat was converted into methyl esters directly and the lower components fractionally separated from the whole bulk, the higher members being separated into liquid and solid acids by Twitchell's lead salt method, methylated and also fractionally distilled. The results are shown in Table II.

## DISCUSSION

As seen from Table I the iodine values of the butter fat of buffaloes receiving cottonseed, sesame, groundnut and hydrogenated groundnut oils have increased by about 6-8 units. This is in general agreement with the observations made by previous workers in the case of cows. Coconut oil and its hydrogenated product have not brought about any

TABLE II  
*Summary of Component Fatty Acids of Buffalo Milk Fat*  
 (Expressed in wt.-%)

Group Dietary fat supplement	I Nil	II Coconut oil	III Cottonseed oil	IV Groundnut oil	V Sesame oil	VI Hydrogen- ated coco- nut oil	VII Hydrogen- ated groundnut oil
Butyric	3.0	2.5	3.5	2.7	3.4	3.0	2.8
Caproic	0.2	0.1	—	—	—	0.2	0.1
Caprylic	1.4	0.6	0.4	0.3	0.4	1.0	0.4
Capric	1.7	1.0	0.8	1.2	1.1	1.2	0.7
Lauric	2.6	7.8	1.1	3.2	1.8	4.2	0.8
Myristic	9.2	12.9	6.2	11.6	9.5	16.4	8.0
Palmitic	37.7	25.4	24.3	22.4	25.3	32.6	26.3
Stearic	11.5	13.9	26.7	16.6	16.1	10.6	20.6
Arachidic	1.3	1.5	1.6	1.0	0.8	1.4	1.6
Behenic	—	—	—	0.8	—	—	—
Total	68.6	65.7	64.6	59.8	58.4	70.6	61.3
Decenoic	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Dodecenoic	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Tetradecenoic	0.8	1.0	0.2	0.6	0.7	1.7	0.4
Hexadecenoic	4.9	5.5	3.1	1.8	3.0	3.3	2.7
Oleic	24.6	26.7	30.7	35.8	36.3	22.1	33.8
Linoleic	0.1	0.3	—	0.3	0.1	0.2	0.1
C <sub>20-22</sub> unsat- urated	0.8	0.6	1.2	1.5	1.3	1.9	1.5
Total	31.4	34.3	35.4	40.2	41.6	29.4	38.7
Sum of acids up to C <sub>14</sub>	19.1	26.1	12.4	19.8	17.1	27.9	13.4

appreciable change in the Reichert value and the saponification equivalents of the butter fat; other fats have lowered the Reichert value and increased the saponification equivalents considerably. These changes can be clearly seen from Table II. The data show that the administration of coconut oil, which contains over 70% of the lauro-myristic acids, has brought about a significant increase in the corresponding group, a marked fall in the palmitic acid and a slight lowering in the other lower saturated acids.

Cottonseed oil, which was fed to group III, has brought about a

reduction in the amount of the lower saturated acids up to palmitic acid, and a considerable increase in the oleic and stearic acid contents. In group IV, the supplementary fat was groundnut oil—a fat richer in oleoglycerides as compared to milk fat. It has lowered the proportion of saturated acids up to palmitic acid, excepting myristic acid. This lowering is counterbalanced by a corresponding increase in the stearic and oleic acid contents. Although the original oil contained about 24% linoleic acid, the amount of the same acid in the resulting butter fat is not altered to any extent. But behenic acid seems to have passed into the milk fat to an extent of 0.8%. This may be due to the fact that the animals were fed a sufficiently large amount of the oil. Hilditch and Thompson (4) had found similar effects in the case of cows fed on rape oil (containing about 50% erucic acid) when erucic acid was found to be present in the butter fat produced. The fifth group of animals was fed on sesame oil supplement which is almost identical with groundnut oil in composition except that it is richer in linoleic acid content. The results obtained are almost similar to those with group IV.

Hydrogenated coconut oil fed to group VI has given results very similar to coconut oil, the proportion of lauric and myristic acids have increased, while palmitic acid has decreased. Hexadecenoic and oleic acid have decreased in the milk fat. This was indicated from the low iodine value of the butter fat. In group VII it is also found that the saturated acids up to palmitic decrease, this lowering being counterbalanced by an increase in the stearic and oleic acids. The increase in the oleic and stearic acid is due to the fact that hydrogenated oil contained about 70% of oleic (and iso-oleic) and about 15% of stearic acid. The increase in the stearic acid content is consistent with the larger amount of this acid in the hydrogenated product.

The similarity in the amount of oleic acid in milk fats of groups III, IV, V and VII indicates the selective absorption of oleic acid as distinct from linoleic acid by the mammary gland. This is clear from the fact that the cottonseed oil, which contains 30% oleic acid as against 50% in the other fats, has brought about an increase in the oleic acid of butter fat to a lesser extent than in other cases.

The remarkable increase in the amount of lauric and myristic acids in groups II and VI by the feeding of coconut oil and its hydrogenated product can be attributed to the fact that these fats are richer in lauro-myristic glycerides (about 70%), thus leading to their selective

adsorption by the mammary glands. Similar results were obtained by Hilditch and Sleightholme (3) as a result of feeding coconut cake to cows and Hilditch and Jaspersen (2) by feeding palm kernel oil. The mechanism of secretion of fat by the mammary glands of buffaloes appear to be almost similar to that of cows. The introduction of the fatty acid glycerides in the form of ingested fats palmitodiolein, steardiolein or triolein interferes with the normal functioning of the mammary gland, thus preventing the production of lower saturated glycerides normal to milk fat of buffaloes when fed with the common edible oils. As compared to cow milk fat (5) buffalo milk fat is characterized by high palmitic and low oleic acid content.

### SUMMARY

1. Buffaloes have been fed on a basal ration supplemented by (a) coconut oil, (b) cottonseed oil, (c) groundnut oil, (d) sesame oil, (e) hydrogenated coconut oil, and (f) hydrogenated groundnut oil. The milk fats from these animals have been analyzed by ester fractionation for the component acids.

2. The characteristics of the supplement were reflected in the milk fat but not quantitatively.

3. Ingestion of coconut oil and its hydrogenated product led to an increase in the corresponding glycerides with a slight diminution in the palmitic glycerides of the ensuing milk fat.

4. Ingestion of groundnut and sesame oils led to an increase in the oleoglycerides of the resulting milk fat. Palmitoglycerides have been lowered considerably in both cases.

5. Cottonseed oil feeding led to a decrease in the amount of lower saturated glycerides and a considerable increase in the amounts of stearic and oleoglycerides.

6. Unlike oleic acid, a high percentage of linoleic acid in the ration did not increase the amount of this constituent in the milk fat.

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# Effect of Sodium Arsenate on Phosphoesterase from Calf Intestinal Mucosa

Charles A. Zittle, Lorna A. Wells and Wm. G. Batt

*From the Biochemical Research Foundation, Newark, Delaware*

Received March 13, 1947

## INTRODUCTION

Enzyme preparations readily obtained from calf intestinal mucosa (1, 2, 3, 4) are capable of hydrolyzing ribonucleic acid, and also desoxyribonucleic acid after treatment with a specific nuclease (4), to nucleosides and phosphoric acid. The hydrolysis involves the break-down of diesters of phosphoric acid, the polynucleotides, and of monoesters, the mononucleotides. Klein (5) found the last stage of hydrolysis to be inhibited by sodium arsenate and utilized arsenate with the above enzyme source to prepare mononucleotides of desoxyribonucleic acid. We found the first stage of the hydrolysis was inhibited to some degree by arsenate also. This led us to follow both stages of the hydrolysis to determine the conditions under which the maximum concentration of mononucleotides would be obtained; the first stage has been followed by the increased solubility of the nucleic acids in the uranium reagent (6) and the second stage by the appearance of phosphoric acid. The procedure utilized for determining phosphoric acid in the presence of arsenate is described. Experiments are reported which bear on the nature of the inhibition by arsenate.

## EXPERIMENTAL

### *Materials*

The preparation of the enzyme has been described (3). The ribonucleic acid was from a commercial source; the desoxyribonucleic acid was prepared (4) by the method of Hammarsten (7) and treated with a specific nuclease (8).

### *Phosphorus Determination*

In general the method of King (9) was used to determine phosphorus. In the presence of arsenic, however, it was found necessary to modify and combine the methods of King (9), Pett (10) and Maver and Voegtlin (11).

*Inorganic Phosphorus.* The samples were first diluted to 10 cc., then 0.1 cc. of concentrated  $H_2SO_4$  and approximately 0.6 g. of sodium bisulfite (measured in a marked tube) were added. The mixture was heated on a water bath at  $55^\circ C.$  for 30 minutes to reduce the arsenate to arsenite. 2.5 cc. of  $H_2O$ , 1 cc. of 2% tartaric acid and 0.22 cc. of concentrated  $H_2SO_4$  were mixed with this solution before addition of the molybdate and sulfonic acid as described by King (9).

*Total Phosphorus.* The samples were evaporated to dryness on a water bath, then digested with 0.35 cc. of concentrated  $H_2SO_4$  and 2 drops of  $H_2O_2$  for 6 minutes. 5 cc. of  $H_2O$  and 5 cc. of 1 N NaOH or 1 N  $NH_4OH$  were added to dilute and reduce acidity of the digest. Bisulfite was added and reduction carried out as above. Development of color was accomplished as for inorganic phosphorus, except that it was necessary to add only 0.16 cc. of concentrated  $H_2SO_4$ .

## PHOSPHORUS,

MG. PER

CC.

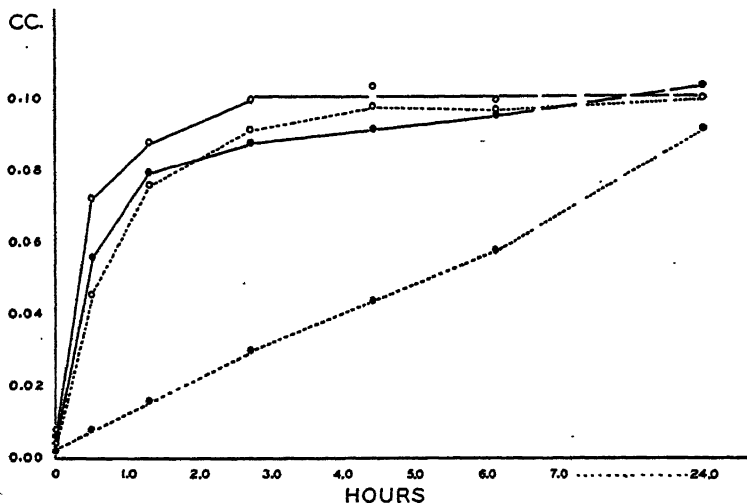


Fig. 1a. Enzymatic hydrolysis of ribonucleic acid, measured by increase in uranium reagent-soluble P and phosphate P, with and without sodium arsenate present. 75 mg. of nucleic acid were dissolved in water and 2.4 cc. of 0.5 M  $NaHCO_3$ . Approximately 10 mg. of the phosphoesterase preparation were added. The final total volume was 47.0 cc. Total nucleic acid P = 0.100 mg./cc. Samples were taken, treated with uranium reagent and prepared for analysis as described (3). Another portion of nucleic acid was handled in the same way with sodium arsenate present (0.00213 M).

— uranium reagent-soluble P  
 - - - phosphate P

○ without arsenate  
 ● with arsenate

Reagent blanks and standard phosphorus samples were run with every series. Full color developed in 5-15 minutes depending on the temperature and age of reagents. Colors were compared in a Klett-Summerson photoelectric colorimeter equipped with filter No. 66.

## RESULTS

The course of hydrolysis of ribonucleic acid with and without arsenate is shown in Fig. 1a, of desoxyribonucleic acid in Fig. 1b.

PHOSPHORUS,

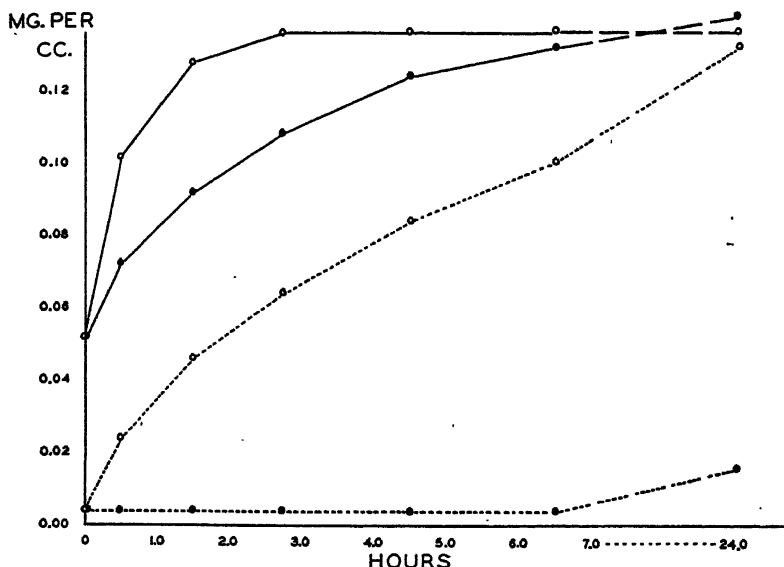


FIG. 1b. Enzymatic hydrolysis of desoxyribonucleic acid, measured by increase in uranium reagent-soluble P and phosphate P, with and without sodium arsenate present. Nucleic acid that had been nuclease-treated (4) was used. The amount of phosphoesterase used was only one-fifth of that used in the experiments for Fig. 1a, otherwise the conditions were the same. Total P = 0.136 mg./cc.

— uranium reagent-soluble P  
 - - - - phosphate P

○ without arsenate  
 ● with arsenate

Experiments were also performed manometrically: the relation between the degree of inhibition and concentration of arsenate with two different concentrations of ribonucleic acid is shown in Fig. 2.



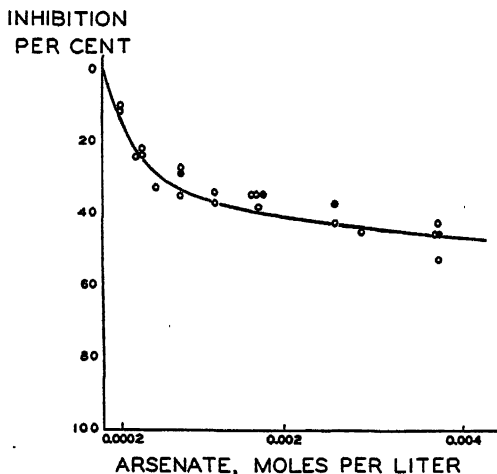


FIG. 2. Effect of sodium arsenate on phosphodiesterase, measured manometrically. The manometric experiments were performed at pH 8.14 as described (3) with ribonucleic acid as substrate. Sodium arsenate was placed in the bottom of the Warburg flasks to give the final concentrations shown and the enzyme (about 1 unit (3)) introduced from the side arm. Retention of  $\text{CO}_2$  (3) by arsenate at this pH value is negligible.

○ concentration of nucleic acid: 20 mg./3.5 cc.

● concentration of nucleic acid: 4 mg./3.5 cc., which is a suboptimal amount (2).

## DISCUSSION

The data in Fig. 1a for ribonucleic acid show that the enzymatic break-down of the mononucleotides of this nucleic acid is inhibited much more than their formation. This procedure, however, would not be efficient for obtaining these mononucleotides and fortunately they can be obtained by hydrolysis of the nucleic acid with alkali at a suitable pH value (12). In desoxyribonucleic acid the pyrimidine nucleotides can be obtained by acid hydrolysis but, because of the lability of the desoxyribose, not the purine nucleotides (13). Fortunately, with this nucleic acid, the hydrolysis of the mononucleotides by phosphoesterase is almost completely prevented by arsenate and the conditions for obtaining a good yield of mononucleotides can readily be seen in Fig. 1b.

Klein (5) believed that arsenate was inhibitory to the stage of enzymatic hydrolysis in which phosphate was released because of the resemblance of arsenate to phosphate, a product of the reaction which

is inhibitory by competition with the substrate. Our manometric studies of the first stage of hydrolysis indicate that arsenate is not inhibitory by competition with the substrate since the percentage inhibition is the same for several different concentrations of nucleic acid (Fig. 2). We have found that phosphate is also inhibitory to the first stage of hydrolysis and in this, just as in the second stage, it appears to inhibit by competition with the substrate (14).

The striking difference in the effect of arsenate on the enzymatic release of phosphoric acid from ribonucleic and deoxyribonucleic acids had been observed by Klein (5). He states that the effect of arsenate on the enzymatic hydrolysis of glycerophosphate and hexosediphosphate is negligible and that ribonucleic acid stands between these compounds and deoxyribonucleic acid. Sodium arsenite up to 0.00257 *M* had no effect on the hydrolysis of ribonucleic acid measured manometrically. It appears that the usual effects of arsenate and arsenite on enzymes, which involve —SH groups, are not concerned here. In view of the large differences with the several substrates a reversible interaction of the arsenate with the substrates may be involved.

Sizer studied (15) the hydrolysis of a number of phosphoric acid esters, none of them mononucleotides, by the phosphatase from bovine intestinal mucosa and other tissues. The hydrolysis was unaffected by most reductants and mild oxidants but was greatly decreased by many strong oxidants giving  $E_h > +400$  millivolts. Sodium arsenate (0.001 *M*,  $E_h = +426$ ), employed with lung phosphatase, however, was not inhibitory, in agreement with Klein's observation (5).

### SUMMARY

The course of hydrolysis of ribonucleic and deoxyribonucleic acids by phosphoesterase from calf intestinal mucosa in the presence of sodium arsenate (0.002 *M*) has been determined. The break-down of both the diesters of phosphoric acid, polynucleotides, and the monoesters, mononucleotides, was inhibited, but the inhibition of the latter greatly exceeded the former. With deoxyribonucleic acid, hydrolysis of the mononucleotides is almost completely prevented, allowing their formation to proceed completely. Manometric studies of the inhibition of the first stage of hydrolysis indicate that this phenomenon is not due to competition with the substrate. The procedure utilized for determining phosphoric acid in the presence of arsenate is described.

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# Metabolism of Pyruvic Acid by Bacteria.

## I. Alterations in Enzyme Activity of Staphylococci when Grown in the Presence and Absence of Glucose

M. G. Sevag and E. Augustus Swart\*

*From the Department of Bacteriology, School of Medicine, University  
of Pennsylvania, Philadelphia 4, Pa.*

Received March 24, 1947

### INTRODUCTION

In a previous study (1) it was found that resting *staphylococci* which had been grown in 0.4% glucose broth oxidized lactate to pyruvate; 92% of the 44% oxidized lactate was isolated as 2,4-dinitrophenylhydrazone of pyruvate. Using *Staphylococcus albus*, 85% of the 12% oxidized lactate was pyruvate. In parallel tests, 97% of the pyruvate added to a suspension of resting cells was recovered unaltered. The cause of the failure of these cells to metabolize pyruvate was then unknown. Some years later (2), it was observed that resting cells of *Staphylococcus aureus*, grown in the absence of glucose, metabolized pyruvate readily. In contrast, when grown in the presence of glucose they failed to do so. This property was restored when the cells were grown in the absence of glucose. Thus, it was demonstrated that cells able to metabolize pyruvate could lose this activity when grown in a medium containing glucose, and could then regain the activity if grown in the absence of glucose. Hence, it may be concluded that the utilization of glucose during growth altered the enzymic make-up of the cells with dependable regularity. In view of the possible occurrence of this phenomenon in other physiological systems it was studied further. The present report concerns this study.

\* Present address: Squibb Institute for Medical Research, New Brunswick, N. J.

## EXPERIMENTAL

*Materials and Methods*

*Staphylococcus aureus*. As indicated in the tables, 13 different strains of *staphylococci*, collected from various sources, were studied. They were kept on extract agar slants. For metabolic experiments, they were transplanted 2-3 times on extract agar every 16-20 hours before inoculating liquid growth media for harvesting sufficient resting organisms.

*Extract Broth*. This medium consisted of 3 g. of beef extract, 10 g. of peptone (Parke Davis) and 5 g. of sodium chloride dissolved in a liter of tap water and adjusted to pH 7.4.

*Streptococcal broth* consisted of the same ingredients contained in extract broth except that sodium chloride was replaced by 10 g. of dibasic sodium phosphate ( $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ ) in a liter of tap water.

*Determination of the weight of bacterial suspensions* was carried out as described previously (3) by standardizing Klett-Summerson photoelectric turbidometric readings against total solids of salt-free bacterial suspensions. Ten ml. of bacterial suspension giving a reading of 66 with No. 54 filter was found to be equivalent to 1 mg. of cocci. In comparative studies, the washed suspensions of various strains were diluted to approximately comparable strength, and calculated volumes of suspensions containing definite weights of cocci per test were used.

*Quantitative Chemical Determinations*. *Pyruvate* was determined gravimetrically as 2,4-dinitrophenylhydrazone according to Simon and Neuberg (4):

*Procedure for Figure 1*. For the determination of pyruvate dismutation 1 mg. of washed organisms was suspended in 10 ml. of pyruvate solution (2 mg./ml.) at pH 7.0 and incubated aerobically at 37°C. for 15 hours. After centrifuging off the organisms, pyruvate was determined gravimetrically as the 2,4-dinitrophenylhydrazone. The hydrazone was completely soluble in dilute sodium carbonate, indicating the absence of a measurable amount of acetaldehyde in the reaction mixture.

*Procedure for Figure 3*. Organisms (grown for 15 hours at 37°C.) were centrifuged and washed twice with *M*/30 phosphate buffer of pH 7.4. For the determination of the rate of dismutation of pyruvate aerobically at 37°C. by these washed cells, each 100 ml. of the reaction systems at pH 7.0 contained 30 mg. of washed cells, 200 mg. of pyruvic acid and 1 mg. of  $\text{Mg}^{++}$  ion in *M*/30 phosphate buffer. Periodically (every 2 hours) aliquots were pipetted from each system and the organisms were centrifuged off. The pyruvate contents of the clear supernatants were determined gravimetrically as the 2,4-dinitrophenylhydrazone.

*Lactate* was determined colorimetrically according to Barker and Summerson (5), and *glucose* colorimetrically according to Sumner (6). The pH of the reaction systems was measured with a glass electrode.

### EXPOSITION AND DISCUSSION OF RESULTS

*Experiments to Determine the Effect of Various Factors.* The results presented in the tables and figures show that *staphylococci* which had been grown in the absence of glucose exercised high activity in metabolizing pyruvate. They failed completely or markedly to do so when grown in the presence of glucose. To account for this difference, the effect of pH in the growth medium, the effect of cocarboxylase on resting cells, and the effect of various growth and enzyme factors on the cells during growth were studied. Similarly, the possible toxic effect of lactic acid produced during growth in a glucose-containing

TABLE I

*Dismutation of Pyruvate by Various Resting Cells of Staphylococcus aureus Which Had Been Grown in Different Media in the Presence and Absence of Glucose*

Strain	Extract broth	Extract broth + glucose	Extract broth +1% phosphate	Extract broth +1% phosphate + glucose	Strepto- coccal broth +1% phosphate	Strepto- coccal broth +1% phosphate + glucose
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
R	100.0	19.7	100.0	43.0	100.0	100.0
S	100.0	10.6	100.0	5.0	100.0	11.0
515	100.0	68.4	100.0	97.5	100.0	97.5
515a	100.0	22.8	100.0	46.1	100.0	97.5
523c	100.0	100.0	100.0	98.0	100.0	91.8
523a	100.0	24.2	100.0	20.2	100.0	53.4
605	100.0	9.1	100.0	6.0	100.0	12.1
606	100.0	26.2	100.0	21.6	100.0	24.2
pH of the culture fluid*	7.0	4.5 to 4.7	7.2	4.7 to 4.9	7.4	4.7 to 5.2

\* pH of all media at 0 hour of growth was 7.4.

The organisms were grown for 15 hours at 37°C. Each reaction system contained 3 mg. of resting organisms /10 ml. *M*/30 phosphate buffer of pH 7.0 containing 19.3 mg. of pyruvic acid and 0.1 mg. of  $Mg^{++}$  ion. After a reaction period of 17 hours at 37°C. the remaining pyruvate was determined as 2,4-dinitrophenylhydrazone.

medium was determined. None of these factors appeared to account for the above mentioned difference of activity.

*Effect of Phosphate Buffer in Regulating the pH during Growth.* With the exception of strain 523c, the staphylococci which had been grown in glucose-containing media were incapable of metabolizing pyruvate completely (Table I). In the presence of additional 1% phosphate, only strains 515, 515a and R appeared to show marked increase of this activity. However, the results of various experiments not reported here, and also the results presented in Table II, show that this effect

TABLE II

*Dismutation of Pyruvate in the Presence and Absence of Added Cocarboxylase by Various Strains of Resting Staphylococcus aureus Grown in Three Different Media. Absence of Correlation Between the Change of the pH of the Culture Fluid During Growth and the Activity of Resting Cells*

Strain	Extract broth + glucose		Extract broth + glucose + 1% phosphate		Extract broth + glucose + 3% phosphate	
	Cocarboxylase added		Cocarboxylase added		Cocarboxylase added	
	None	2 $\gamma$ /ml.	None	2 $\gamma$ /ml.	None	2 $\gamma$ /ml.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
R	33.1*	62.5	48.9	76.5	70.9	100.0
S	6.5*	10.0	0.9	3.7	4.6	4.8
P78	3.7*	4.6	6.8	14.1	8.0	28.2
PGH	5.5*	8.1	7.1	13.3	20.7	39.5
515	47.4**	59.1			32.1	57.4
515a	18.1**	21.1			60.3	73.6
523a	28.8**	39.5			24.3	34.6
523c	99.5**	98.8			32.4	46.9
Rose	29.9**	34.6			19.2	21.6
616	8.0**	9.0			5.5	6.5
604	0**	10.7			0	14.8
605	0**	0			0	0
606	25.5**	26.0			17.7	32.0
pH of culture fluid***	4.3 to 5.0		4.7 to 5.8		6.4 to 6.9	

\* Average of the results of 3 experiments.

\*\* Average of the results of 2 experiments.

\*\*\* pH of the media at 0 hour of growth was 7.4.

of phosphate is not constant. As will be seen at the bottom of Table I, the amount of phosphate present was not sufficient to maintain the pH of the culture at neutrality when glucose was present. Increasing the concentration of phosphate to 3%, approximate neutrality (pH 6.4 to 6.9) throughout the growth period was maintained. However, as can be seen in Table II, the cells harvested from glucose-containing media were as inactive as those harvested from cultures which showed a pH variation from 7.4 to 4.3-5.0.

TABLE III

*Failure of Various Growth Factors to Affect the Pyruvate Dismutating Activity of Staphylococcus aureus (805) when Grown in the Presence of Glucose*

Basal Medium to which were added	Bacterial turbidity readings	Pyruvate dismutation by resting cells
		<i>per cent</i>
1. Control	166	12.6
2. Thioglycollate (500 $\gamma$ /ml.)	185	13.9
3. Thioglycollate (1000 $\gamma$ /ml.)	185	27.8
4. Methionine (500 $\gamma$ /ml.)	200	10.7
5. Methionine (1000 $\gamma$ /ml.)	160	10.2
6. Cysteine (500 $\gamma$ /ml.)	195	9.1
7. Cysteine (1000 $\gamma$ /ml.)	172	10.7
8. Glutathione (500 $\gamma$ /ml.)	190	9.1
9. Sulfathiazole (1000 $\gamma$ /ml.)	23	5.4
10. Thiamine chloride (1000 $\gamma$ /ml.)	195	0
11. Adenylic acid (50 $\gamma$ /ml.)	55	0
12. Adenylic acid+thiamine chloride (1000 $\gamma$ /ml.)	190	0
13. Adenosine triphosphate (50 $\gamma$ /ml.)	85	0
14. Adenosine triphosphate+thiamine chloride (1000 $\gamma$ /ml.)	195	0
15. Cocarboxylase (10 $\gamma$ /ml.)	100	0
16. Basal medium	121	12.8
17. Basal medium (without glucose)	23	91.5

*Basal medium:* 1% vitamin-free casein hydrolyzate + salts + tryptophan (20  $\gamma$ /ml.) + glucose (0.5%) + nicotinamide (1  $\gamma$ /ml.) + thiamine chloride (1  $\gamma$ /ml.) + cystine (50  $\gamma$ /ml.).

*Growth:* Bacterial turbidities were measured with a Klett-Summerson photoelectric colorimeter after a growth period of 16 hours. 10 ml. of bacterial turbidity with a reading of 66 contained 1 mg. of cocci.

*Pyruvate dismutation:* Weight of washed resting cells used per system was 1 mg. in a 10 ml. volume containing 18.7 mg. of pyruvate.



*Effect of Cocarboxylase on Resting Cells.* The effect of addition of cocarboxylase to the reaction systems was studied to determine whether the partial or complete loss of carboxylase activity, during growth in a glucose-containing medium, was due to destruction of the coenzyme, or to the loss of other critical enzyme factors. The results presented in Table II show, however, that this coenzyme was incapable of exercising increased activity on those cells which were practically inactive. Those cells which showed partial activity (strains R, 515 and 523a) showed slightly increased activity when cocarboxylase was included in the system.

*Effect of Various Substances on Growing Cells.* The results presented in Table III show that none of the substances of vitamin and coenzyme nature, or others, altered the above discussed effect of glucose on growing cells.

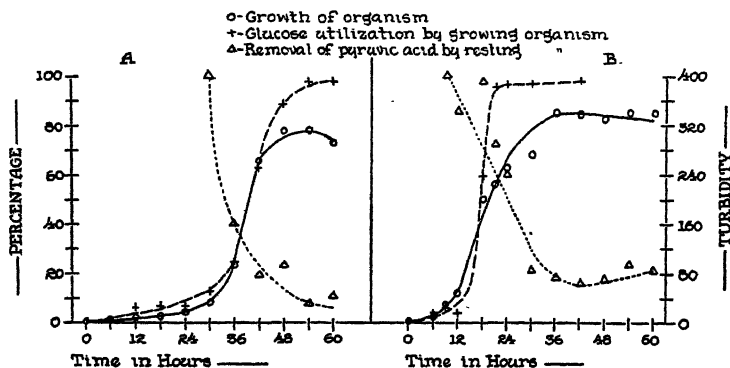


FIG. 1. Comparison of the rate of growth and glucose utilization during growth with the dismutation of pyruvate by the washed, resting cells of *Staphylococcus aureus* (strain 523c) harvested at various periods of growth.

The 523c strain of *Staph. aureus* was grown at 37°C. in 1% vitamin-free casein hydrolyzate (SMACO) containing  $\text{KH}_2\text{PO}_4$  (4.5 g./l.) and adjusted to pH 7.4 with a solution of sodium hydroxide, Mohr's salt (0.008 g./l.), magnesium sulfate (0.04 g./l.), cystine hydrochloride (0.05 g./l.), thiamine chloride (1  $\gamma$ /ml.), nicotinamide (1  $\gamma$ /ml.), tryptophan (20  $\gamma$ /ml.) and glucose (5 mg./ml.). During growth, periodically samples of culture were pipetted and the organisms centrifuged off and washed with *M*/30 phosphate buffer of pH 7.4. Using aliquots of the supernatants, glucose was determined colorimetrically.

The medium for growth was the same as for Fig. 1-A with the addition of the following vitamins: 1.0  $\gamma$ /ml. each of calcium pantothenate, riboflavin, and 0.1  $\gamma$  of biotin/ml. and 0.01  $\gamma$  of crystalline *L. casei* factor/ml. of medium.

*Rate of the Decline of Activity during Growth of Staphylococci in Parallel with the Utilization of Glucose.* Failing to demonstrate a direct relationship between the above mentioned factors and the failure of *staphylococci* to metabolize pyruvate after growth in the presence of glucose, attention was directed to the study of the earliest period of growth when the cells were active and the period when this activity began to decline. Strain 523c was chosen for this particular study when it was noted that this organism required a long induction period preceding growth. The results obtained from this strain, grown in the presence and absence of certain additional vitamins, are plotted in Fig. 1. After an induction period of from 24 to 36 hours the growth was fairly rapid. It can be seen (Fig. 1A) that at the start of rapid growth the cells metabolized pyruvate completely. Concomitant with this activity the quantity of glucose used was about 10% of the total amount. The cells which were produced under these conditions were apparently as active as those produced in the absence of glucose. Rapid decline in the metabolism of pyruvate occurred when the resting cells were harvested at the time of accelerated glucose utilization. At the end of 48 hours glucose was almost completely used up, growth had stopped and the pyruvate-metabolizing activity of harvested resting cells was 10% of its activity at the beginning of glucose utilization. Fig. 2 (523c) also shows that there was a gradual increase in the pyruvate activity of the cells at a time (36-hour period) when glucose utilization was at a minimum (from 10 to 20% of the total amount). Following this phase glucose was actively utilized and the capacity of the washed cells to metabolize pyruvate sharply declined.

In the presence of additional vitamins (Fig. 1B) (pantothenate, riboflavin, biotin and crystalline *L. casei* factor) the long induction period was eliminated. Here again the maximum pyruvate metabolic activity of the cells was observed (10-hour growth period) when only less than 5% glucose was utilized. Following this period glucose utilization increased abruptly. Concomitant with this, the pyruvate activity of the resting cells fell, respectively, to 50% (24 hours) and 10% (36 hours) of the 100% activity observed at the beginning of glucose utilization. These results showed definitely that the decline in the pyruvate-metabolizing activity of the resting cells occurred simultaneously with the increased glucose utilization during growth.

*Determination of Lactic Acid Formed during Growth in Relation to the Pyruvate Activity of Resting Cells.* Since lactic acid is one of the principal

reaction products of glucose metabolism, an inquiry was made concerning the possible effect of lactic acid on the pyruvate activity of cells harvested from such an environment. The results with strains 523c and 605 showed that sharp decline in pyruvate activity occurred when there was only a measurable amount of lactic acid formed. Under these conditions the concentration of lactic acid ranged from  $1.3 \times 10^{-2} M$  to  $7.27 \times 10^{-2} M$  for strain 523c at 36- and 48-hour periods, and from  $3.0 \times 10^{-3} M$  to  $1.8 \times 10^{-2} M$  at 12- and 18-hour periods for strain 605. It does not appear that lactic acid was a critical factor in this respect.

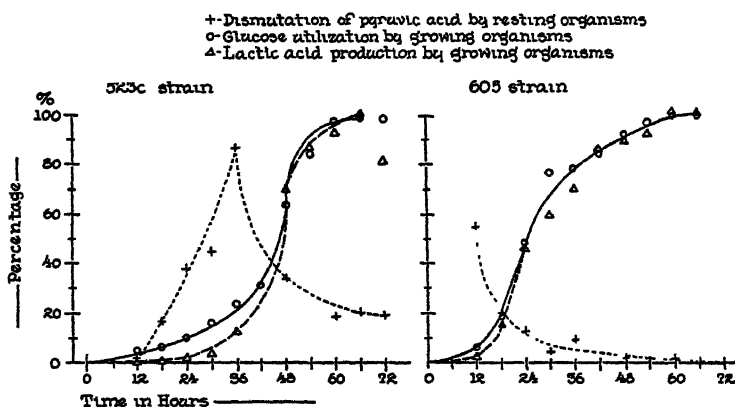


Fig. 2. Comparison of the glucose utilization and lactic acid formation by growing organisms with the dismutation of pyruvate by the washed, resting cells of *Staphylococcus aureus* (Strains 523c and 605) harvested at various periods of growth.

The medium used for the growth of strains 523c and 605 was identical with that described under Fig. 1-A.

*Formation of Lactic Acid, during Growth in Casein Hydrolyzate, in Amounts Greater Than Required by Theory.* The results presented in Table IV show that strain 523c produced 1.75 and 3.28 moles of lactic acid, during 36 and 54 hours, respectively, and strain 605 produced 1.55 and 3.68 moles during 12 and 24 hours, respectively. Since only 2 moles of lactic acid are obtainable from one mole of glucose, the extra moles of lactic acid must have been derived from amino acids. Since, in the absence of glucose, there was no lactic acid formation from amino acids, the extra lactic acid production in the presence of glucose must have involved interactions between glucose and amino acid oxidation

TABLE IV

*Rate of Lactic Acid Formation in Excess of the Theoretical Amounts Obtainable from Glucose During the Growth of Staphylococcus aureus*

Strain 523c				
Period	Mg. staphylococci /liter culture	Moles of glucose used	Moles of lactic acid formed	<u>Lactic acid</u> Glucose
hr.		$\times 10^{-3}$	$\times 10^{-3}$	
12	13.6	1.40	0.11	0.08
18	15.1	1.94	0.33	0.17
24	24.2	3.06	1.78	0.58
30	27.2	5.00	3.67	0.73
36	60.6	7.50	13.11	1.75
48	380.0	20.56	72.78	3.54
54	606.0	27.33	89.56	3.28
60	681.0	31.44	96.67	3.07
66	621.0	31.89	104.44	3.28
Blank	—	32.50	—	—

Strain 605				
		$\times 10^{-3}$	$\times 10^{-3}$	
12	44.0	1.94	3.00	1.55
18	120.0	5.83	18.44	3.16
24	275.5	15.39	56.78	3.68
30	366.6	24.56	72.78	2.96
36	530.3	25.17	85.67	3.40
42	590.9	27.06	104.00	3.86
48	638.0	29.50	109.56	3.71
54	638.0	31.17	112.22	3.60
60	681.0	31.89	122.33	3.83
66	621.2	31.94	122.33	—
Blank	—	32.22	—	—

products. In experiments over a 20-hour period with resting cells grown in glucose-containing media, the effect of glucose on the formation of lactic acid from pyruvic acid was determined. When the system contained 20.3 mg. of pyruvate alone, 31% of the pyruvate was metabolized yielding 1.8 mg. of lactic acid. When the system contained 32.3 mg. of glucose alone, 90% of the glucose was metabolized yielding 13.1 mg. of lactic acid. When glucose and pyruvate were present in the same system, both were quantitatively removed, yielding 26.1 mg. of

lactic acid. These results indicated that pyruvate was completely reduced to lactate due to glucose metabolism. Correlating the results obtained from these resting cell experiments with the results presented in Table IV, it would appear that  $\alpha$ -keto acids resulting from the deamination of amino acids were reduced to  $\alpha$ -hydroxy acids by certain intermediary products of glucose metabolism during growth, unless a substance was produced during growth which interfered with the colorimetric determination, yielding higher lactic acid values than could be accounted for by the amount obtained from glucose.

*Determination of the Rate of Pyruvate Metabolism by Resting Cells Grown in the Presence and Absence of Glucose.* Up to this point the results did not indicate the rate or the type of pyruvate metabolism. To investigate the effect of the presence or absence of glucose in a given growth medium on the activity of resting cells harvested therefrom, the kinetics of pyruvate metabolism were determined. Results of these experiments are plotted in Fig. 3. It can be seen that the washed cells harvested from a glucose-free medium (curve II) metabolized completely a given amount of pyruvate within a reaction period of 8 hours. In contrast (curve IV), the cells from a glucose-containing medium metabolized about 80% of pyruvate during a period of 48 hours. The results with strain 523c qualitatively presented the same pattern. Postulating the presence of the same enzyme systems in cells harvested from glucose-free growth media and media containing glucose, it would mean that the cells are qualitatively identical, but quantitatively differ in the rate of pyruvate metabolism. The slow rate of reaction characteristic of the cells grown in glucose medium might be attributed to an alteration in the permeability of the cell to pyruvate, providing intracellular enzymes are involved. This might be considered as a plausible explanation if we could also demonstrate structural chemical differences to account for rapid and slower permeability of cells to pyruvate.

On the other hand, the rapid and slow pyruvate metabolism could represent two types of reaction mechanisms. This necessitates the demonstration that the reaction products of pyruvate metabolism qualitatively or quantitatively are sufficiently dissimilar to account for the observed difference. This would mean that the enzyme make-up of the cells harvested from the two media is different. The preliminary results recently obtained in this laboratory by Dr. Olive E.

McElroy seem to lend support to the latter explanation. It may here be stated briefly that the cells grown in the absence of glucose dismutate pyruvate and those grown in the presence of glucose metabolize pyruvate oxidatively primarily, though they show a small dismutative activity.

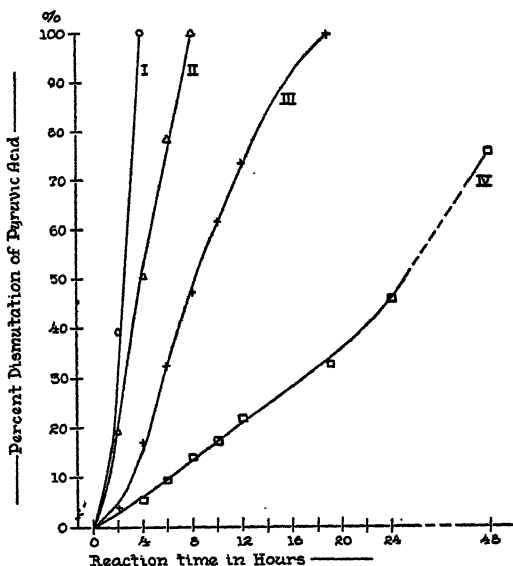


FIG. 3. The rate of dismutation of pyruvate by washed resting *Staphylococcus aureus* (strains 523c and 606) grown in various media.

Curve I. Strain 523c grown in extract broth.

II. Strain 606 grown in extract broth.

III. Strain 523c grown in extract broth containing 0.5% glucose.

IV. Strain 606 grown in extract broth containing 0.5% glucose.

### SUMMARY AND CONCLUSION

Resting cells of various strains of *Staphylococcus aureus*, which had been grown in a medium containing no glucose, metabolized pyruvate completely during a period of from 2 to 8 hours. Those grown in the presence of glucose have been found to metabolize pyruvate to only a small degree, or failed completely to do so during a greater reaction period of 20 hours. The addition of cocarboxylase to the reaction systems occasionally increased the activity of partially inactive cells

but it was ineffective when added to systems containing totally inactive resting cells.

The maintenance of the medium at neutrality throughout the growth period, the addition of thioglycollate, methionine, cysteine, glutathione, thiamine, cocarboxylase, adenylic acid, adenosine triphosphate and sulfathiazole to the growth medium, did not alter the above observed effect of glucose on the growing cells. The amount of lactic acid produced during growth did not appear to affect the activity of the cells in this respect. (During the growth in casein hydrolyzate there was produced about twice as much lactic acid as could be accounted for by the amount of glucose utilized. This extra amount apparently resulted from interactions between metabolic products of glucose and amino acids. No lactic acid was produced in casein hydrolyzate when growth took place in the absence of glucose.)

The cells harvested from glucose-containing media from various stages of growth manifested greatest activity to metabolize pyruvate when glucose metabolism was minimal. The decline in the activity of the harvested cells seemed to parallel the rise in the utilization of glucose during growth. With complete utilization of glucose the activity of the harvested resting cells was practically absent.

The cells harvested from glucose-containing media metabolized pyruvate at a very slow rate during the first 24-hour period, metabolized it 80–100% after a period of 48 hours. This is interpreted to show that the metabolism of pyruvate by these cells was different from those derived from glucose-free medium (the reason for the slow rate of reaction requiring 48 hours is as yet unknown). Unpublished results recently obtained in this laboratory show that the cells derived from glucose-free media *dismutate* pyruvate yielding *one* mole of lactic acid and *one* mole of acetic acid per *two* moles of pyruvic acid metabolized. On the other hand, the cells derived from a glucose-containing medium principally *oxidized* pyruvic acid, yielding, on the average, *two* to *four* moles of acetate for *one* mole of lactate produced. It thus appears that these cells possess only about 20% of their original dismutative activity.

The results reported here were obtained in aerobic experiments without resorting to manometric measurements of the oxygen consumed and carbon dioxide evolved. In previous anaerobic manometric measurements during a period of four hours, the resting cells grown in

the presence of glucose failed to liberate carbon dioxide from pyruvate (2). We plan to study, also manometrically, the possible difference in the aerobic oxidation of pyruvate by resting cells grown in the presence and absence of glucose.

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# The Conversion of Carotene to Vitamin A in the Fish

J. B. Neilands

*From the Atlantic Fisheries Experimental Station, Halifax, Canada<sup>1</sup>*

Received March 24, 1947

## INTRODUCTION

One of the most vital problems in the vitamin A field at present is that of the identification of the site of conversion of carotene to vitamin A. If the mechanism of the conversion is *via* the carotenase of Olcott and McCann (1) it would seem logical to search for this enzyme in certain fish tissues since most species of fish have large stores of the vitamin in the liver, intestinal wall, etc. However, it is not definitely known (Drummond and Hilditch (2)) whether the cod, for example, obtains its vitamin A as such by eating some smaller organism or whether the vitamin is derived from carotene precursors in the food.

Morton and Creed (3) claim to have converted carotene to vitamin A in two fresh water species but, so far as the author is aware, this study has not been extended to marine fish or shellfish. The following experiments were undertaken to test for the conversion, if any, in Atlantic cod (*Gadus callarias*) and in the lobster (*Homarus americanus*).

## EXPERIMENTAL

The fish and shellfish were kept in tanks fed by a continuous flow of clean sea water. They were segregated into two groups one being fed a carotene diet and the other a plain diet. At the start of the experiments a number of individuals were assayed for initial levels of vitamin A. The carotene concentrate fed contained about 3,000 mg.-%  $\beta$ -carotene and had been prepared by ether extraction of dehydrated cereal grass.<sup>2</sup> Since it has been shown by Moore (4) that vitamin E facilitates the utilization of carotene, the latter was dissolved in cottonseed oil, the control group

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<sup>1</sup> Present address Dept. of Biochemistry, Univ. Wisconsin, Madison.

Taken from a thesis submitted to Dalhousie Univ., Halifax, Canada, for the degree of Master of Science.

<sup>2</sup> Received from W. R. Graham, Cerophyl Lab., Inc., Kansas City, Mo.

receiving the oil without the added carotene. No attempt was made to administer the provitamin quantitatively. For the lobsters the food was prepared by homogenizing a quantity of the carotene in oil, some white cod muscle and distilled water in a Waring blender. The cod received pieces of frozen white haddock (*Melanogrammus aeglefinus*) muscle which had previously been injected with a charge of carotene-oil or with the plain oil (control group).

At the conclusion of the experiments the fish were frozen and the vitamin stores of certain tissues determined. The tissues for assay were saponified by the method of Jensen and With (5). It was found necessary when assaying the eyes to subject them to grinding in a mortar containing clean sand. The vitamin analyses were carried out with a Photovolt absorptiometer, Model 410. This instrument was found to give excellent agreement with Carr-Price values assayed here and elsewhere.

## RESULTS

Table I suggests that the cod may have the power to convert carotene to vitamin A. Since there seemed to be relatively little correlation between body weight and liver weight it was decided to record the vitamin content as per gram of fish.

TABLE I

*Results of Feeding Cod (Gadus callarias) Diets with and without Carotene*  
(Experimental period. . . 3 months)

(a) Diet: feeding naturally on Grand Banks	
Weight of individual fish g.	Liver vitamin A/g. of fish <i>Int. units</i>
580	10.12
682	19.55
875	11.21
1124	10.43
1136	22.53
1241	26.07
1352	14.08
Av. 15.88 $\pm$ 5.6 *	
(b) Diet: without carotene	
Weight of individual fish g.	Liver vitamin A/g. of fish <i>Int. units</i>
366	9.24
548	19.78
810	4.89
1038	9.30
1040	31.26
1119	28.68
1175	29.21
Av. 18.77 $\pm$ 9.6 *	

\* Standard errors.

TABLE I—*Continued*

(c) Diet: with carotene

Weight of individual fish g.	Liver vitamin A/g. of fish <i>Int. units</i>
273	16.83
366	16.45
409	24.13
425	15.59
598	39.45
1291	53.26
1437	43.16

Av. 29.84 ± 14. \*

Although initial values for the vitamin content of the lobster eyes (Table II) were not determined the difference between the two groups seems particularly striking.

## DISCUSSION

The experimental evidence favors the theory of sluggish conversion of the provitamin and substantiates our present knowledge of the extreme stability of vitamin A in certain fish tissues. Pugsley *et al.* (6) have shown that the total vitamin in the liver of the cod does not change with the spawning season although the units/g. of oil may rise to very high levels in this period because of loss of liver fat.

TABLE II

*Results of Feeding Lobster (Homarus americanus) Diets with and without Carotene*  
(Experimental period. . . 48 days)

(a) Diet: feeding naturally

Weight of individual specimen g.	Vitamin A/g. of hepato-pancreas <i>Int. units</i>
396	68.0
398	83.0
400	63.9
401	73.3
431	91.2
444	97.2
459	83.1
498	97.2
501	95.0
522	87.6

Av. 83.9 ± 11.5 \*

\* Standard errors.

TABLE II—*Continued*

(b) Diet: without carotene

Weight of individual specimen <i>g.</i>	Vitamin A/g. of hepato-pancreas <i>Int. units</i>	Vitamin A/g. of eyes <i>Int. units</i>
369	23.9	95.1
380	36.8	106.9
388	57.0	98.6
389	37.5	109.3
411	50.5	106.6
431	26.9	91.8
436	42.7	108.3
448	55.4	97.9
454	37.1	94.1
480	33.2	98.0
489	21.9	106.3
504	22.3	101.9
514	27.0	94.8
535	31.1	99.7
549	20.0	97.4
601	42.5	93.4
Av. 35.9 $\pm$ 11.0*		Av. 100.0 $\pm$ 5.6*

(c) Diet: with carotene

Weight of individual specimen <i>g.</i>	Vitamin A/g. of hepato-pancreas <i>Int. units</i>	Vitamin A/g. of eyes <i>Int. units</i>
417	83.7	192.1
430	44.8	173.7
431	58.3	353.0
450	60.5	231.2
462	47.5	161.4
482	42.7	189.0
482	35.2	119.6
488	84.6	143.4
489	35.2	152.2
493	46.2	135.9
501	43.5	196.3
504	36.8	141.6
522	57.7	157.1
525	42.9	275.1
541	72.3	139.5
581	61.5	173.2
Av. 53.3 $\pm$ 15.4*		Ave. 183.3 $\pm$ 58.1*

\* Standard errors.

It should be stressed that in these experiments considerable variation existed between values obtained for individual specimens. The use of larger numbers of animals would be highly desirable. Efficient conversion in the fish may be rather specifically associated with the general nutrition of the animal organism, absorption of the provitamin, or other related phenomena.

#### SUMMARY

The Atlantic cod (*Gadus callarias*) and the lobster (*Homarus americanus*) appear to convert carotene to vitamin A. The conversion is sluggish under the experimental conditions employed.

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# A Study of the Isolation of Blood Group A-Specific Substance from Commercial Hog Gastric Mucin and Some Observations on the Separation of A-Substance from Other Natural Sources<sup>1</sup>

David H. Brown, Edward L. Bennett, George Holzman  
and Carl Niemann

*From the Gates and Crellin Laboratories of Chemistry, California  
Institute of Technology, Pasadena, California<sup>1a</sup>*

Received September 9, 1946

## INTRODUCTION

The serological properties of blood group A-specific substances<sup>2</sup> isolated from a variety of sources (for bibliography, see (1)) are the most characteristic features of these preparations, and, consequently, immunological techniques have been relied upon to evaluate starting materials and preparative procedures. However, the data of previous investigators have permitted only limited comparisons to be made of the efficacy of different procedures for isolating A-substance (2, 3). This situation, which has been commented upon before (3), exists because of the variation in the methods used by different authors in carrying out the serological tests and the unpredictable degree to which

<sup>1</sup> This work was done in part under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the California Institute of Technology.

<sup>1a</sup> Contribution No. 1085.

<sup>2</sup> By "group A-specific substance" here, is meant any material effective in inhibiting isoagglutination of human type A erythrocytes by type B serum and also effective in inhibiting lysis of sheep erythrocytes by human A-cell immune rabbit sera. Evidence has recently been presented (23) that two different group-specific substances are obtainable from human urine of type A individuals, and that one of these materials is effective in inhibiting hemolysis of sheep erythrocytes by immune rabbit sera while the other is active in inhibiting isoagglutination of human A cells. We have no evidence for the existence of two such different group-specific substances. We have not examined urine, however, as a source of group-specific materials.



such tests are influenced by changes in experimental details. Hence, we believe that questions involving the usefulness of various natural products as starting materials for the preparation of A-substance and, particularly, of the most advantageous procedure for effecting its isolation can best be answered by the isolation of a large number of A-substance preparations and by the parallel testing of these preparations by several serological procedures. Accordingly, we have undertaken a comparative study of the preparative procedures described in the literature (for references, see Table I). Most of our work has been done with commercial hog gastric mucin, a material in which A-substance has long been known to be abundantly present. By these experiments we have been able to secure definite evidence bearing on the question of the similarity of A-substance preparations isolable in different ways from hog mucin, and it has been possible to determine which of the several preparative methods that have been proposed is the most satisfactory from the standpoint of yield and potency of product. It has also been possible to compare A-substance preparations obtained from other sources with those obtained from hog gastric mucin.

#### EXPERIMENTAL

*Serological and Other Analytical Procedures.* Two serological tests have been used. The *inhibition of hemolysis test*, which will be discussed in detail shortly (8), makes use of a colorimetric determination of the extent of lysis of sheep erythrocytes by human A-cell immune rabbit sera in the presence of guinea pig serum as a source of complement. The precision of the hemolysis test is high, and the results obtained by it can be used to distinguish preparations which differ by only 10-20% in serological activity. Each of the fractions obtained has also been assessed by an *inhibition of isoagglutination test*, using the same lot of human type B serum throughout, and using pooled human type A erythrocytes. In this latter test preparations must differ in activity by approximately 100% to be distinguishable. A detailed description and discussion of the inhibition of isoagglutination test has been given (9). We have also measured the amount of color given by each fraction when treated, after alkaline digestion, with a *modified Ehrlich reagent* (*p*-dimethylaminobenzaldehyde) (9). The intensity of color obtained with this reagent after heating blood group A-specific substance with alkali has been found to be correlated with the isoagglutination in-

hibition titers of the specific substance preparations (9). Since the existence of the correlation is dependent in part upon differences in preparations detectable by means of the inhibition of isoagglutination test, only very little correlation is demonstrable for the relatively similar and potent preparations discussed below. However, it has been found that for these preparations there exists a marked correlation between the results of the color test and of the relatively precise inhibition of hemolysis test. For this reason the color produced with Ehrlich's reagent is interpreted as a characteristic property of blood group A-specific substance.

*The Separation of A-Substance from Hog Gastric Mucin.* The general isolation procedures employed were: 1, sodium sulfate fractionation from aqueous solution (2); 2, alcohol fractionation from aqueous solution (3); 3, alcohol fractionation from a 90% phenol solution (2); 4, electrodialysis followed by alcohol fractionation; 5, a combination of two or more of the foregoing procedures. Table I contains abbreviated descriptions of the method of isolation of each fraction that is considered in this communication.

*The Separation of A-Substance from Human Erythrocytes.* The alcohol extraction procedures of Hallauer (7) and of Kossjakow (10) have been followed, with large amounts of dried cells and with dried stromata obtained by centrifuging aqueous suspensions of cells from which hemoglobin had been removed by repeated washing. Material extracted by boiling absolute ethanol from dry stromata (7), fraction 45 (see Table I), as well as fractions arising in a similar way from whole erythrocytes or arising from either source by extraction with 95% ethanol in the cold, has nearly as much potency in inhibiting isoagglutination and in inhibiting hemolysis as any of the other fractions arising from the alcohol extraction procedure of Hallauer (7). This ethanol-soluble material is obtained as the major part of all material extracted from dry stromata (7% yield) and was discarded by Hallauer. Because of the insolubility of the material in water, suspensions of it were prepared for serological testing by dilution of an absolute alcohol solution with physiological salt solution. The activity of fraction 45 in inhibiting isoagglutination and in inhibiting hemolysis is about 500 times less than that of the highly active preparation, fraction 66 (see Table I), from hog gastric mucin. Several other materials (see Table I) have been obtained by alcohol extraction in 0.2-0.3% yield from lyophilized erythrocytes and these fractions have all had from 150 to 300 times less activity in the inhibition of hemolysis test and from 500 to 1,000 times less activity in the inhibition of isoagglutination test than a potent preparation from hog mucin. Digestion of stromata with crude pancreatin has given a fraction, 161, obtained in 10% yield, which has the same order of activity as fractions obtained by alcohol extraction from erythrocytes.

The extremely low yield and serological activity of group A-specific substance isolated from human erythrocytes is illustrated by the data in Table I (cf. also Bray, Henry and Stacey (11)). This situation may be due to the relatively minute concentration of active material in erythrocytes or to the possibility that the procedures we have used in extracting it are relatively ineffective. This latter possibility is sug-

gested by recent experiments of Calvin and coworkers (12) which are in agreement with earlier observations of Schiff and Adelsberger (13) and Landsteiner and van der Scheer (14). It is probable that our alcohol-soluble fraction 45 obtained as a major fraction from stromata is similar to these lipid-rich fractions obtained by other workers. The similarity in yield of preparations with comparable activity from stromata and from whole erythrocytes is evidence that a large part of the active material was retained in the course of lysing the erythrocytes and washing the insoluble residue. This is in contrast with what might be expected from the results reported by Calvin *et al.* (*loc. cit.*). Further evidence is given by the experiments of Belkin and Wiener (15) who found that the titer of A-substance in their stromata preparations was higher than that in the parent erythrocytes and that the ratio of these titers was inversely proportional to the yield of stromata obtained. Thus, the unsatisfactory yield of A-substance from human erythrocytes is most probably attributable not to the inefficacy of the isolation procedures but rather to the very small amount of active material present.

*The Separation of A-Substance from Human Ovarian Cyst Fluids.* According to the observations of Yosida (16) and the extensive investigations carried out by Morgan and Van Heyningen (17), King and Morgan (18), and Morgan and Waddell (19), the fluid from pseudomucinous ovarian cysts may contain a blood group-specific substance in water-soluble form which has similar serological specificity to that present in the stromata of the individual's erythrocytes. We have examined such cyst fluids, and from one containing A-specific substances (Fluid No. 162, Table I), have separated two preparations by alcohol fractionation. One of these, fraction 113, precipitated by 50% (v/v) ethanol, has about one-third the potency of the native cyst fluid in inhibiting hemolysis; the other, fraction 119, precipitated by 66% (v/v) ethanol, has about twice the potency. It may be significant that fraction 119 has as much anti-hemolytic activity as some of the less pure fractions, 109 and 115 (see Table I), from hog gastric mucin, and yet has 4-6 times less potency in inhibiting isoagglutination than these fractions. Morgan and Van Heyningen report (17) no such discrepancy in the activities of two materials isolated from cyst fluids by rejection of material soluble in 90% phenol.

*The Specific Substance in Commerical Hog Pepsin.* We have isolated from Wilson pepsin (1:10,000, soluble powder), in the small yield of 1-2%, fractions 22 and 26 which are extremely potent in inhibiting hemolysis, one of which, fraction 22 (see Table I), also has a high degree of activity in inhibiting isoagglutination. These materials are more active as antihemolytic factors than any other substance we have obtained directly from any source. Only fractions 47 and R.7 F.5A, obtained from hog gastric mucin by other than the usual mild procedures (see Table I and the discussion below), have activities nearly or equally as high. Fractions 22 and 26 do not exhibit the augmented equivalent N-acetylglucosamine content of the somewhat degraded preparations 47, 48, R.6 F.5A, and R.7 F.5A and give somewhat less color in the modified Ehrlich procedure (9) than our highly active material from mucin, fraction 66. In the case of fraction 22, obtained according to Landsteiner and Chase (6) by alcohol fractionation of an aqueous solution of pepsin previously heated at pH 6 and pH 3.5, there is no indication of extensive degradation, judged from its still relatively high inhibition of isoagglutination titer. This titer of fraction 26, however, is not

commensurate with its antihemolytic activity and, thus, the autolysis procedure of Landsteiner and Harte (3) must be regarded as bringing about some degradation of the specific substance. It appears, then, that the specific substance isolable from pepsin (fractions 22 and 26) differs from that obtained from hog gastric mucin (fraction 66), 1, in having a lower equivalent per cent N-acetylglucosamine, 2, in having a somewhat diminished inhibition of isoagglutination potency, and 3, in having enhanced activity in inhibiting hemolysis. The extent to which these properties are related is unknown. When the serological properties of the fractions from pepsin are considered together with their behavior in the modified Ehrlich procedure, it is apparent that these specific substances partially resemble the somewhat degraded preparations, 47 and R.7 F.5A, whose preparations are discussed below. A preparation of Parke, Davis and Co. pepsin (1:3,000, granulated) proved to be valueless as a source of A-substance, confirming the findings of Freudenberg *et al.* (20) that different preparations of pepsin vary widely in their content of group A-specific substance. The Fairchild Bros. and Foster, 1:15,000, pepsin used by Landsteiner and co-workers in their isolation studies (3, 6) would appear to have the highest concentration of A-substance of any commercial preparation.

## DISCUSSION

The data in Table I indicate that, irrespective of the method of isolation used, no fraction was obtained from hog gastric mucin which had more than 2-4 times the activity in the inhibition of isoagglutination test or 2-3 times the activity in the inhibition of hemolysis test of a centrifuged (Sharples), undialyzed suspension of the original mucin (fractions 62 and 135, Table I). The inhibition of hemolysis potency of the most active fraction derived by application of any one isolation procedure alone was the same, within 20%, as the potency of one obtained by any other procedure (*cf.* fractions R.1 F.2A, R.2 F.2A, R.3 F.2, 87, R.5 F.3, 128, 143, 120, 31, 66, 69, Table I), which might be taken as evidence that the active material obtained is a fairly well-defined preparation, although not necessarily homogeneous. It is also apparent from the data of Table I that, within this group of similar fractions, significant differences occur with respect to the equivalent N-acetylglucosamine content<sup>3</sup> (9), and even with respect to the serological activity itself, indicating that preparations of A-substance

<sup>3</sup> Various values can be found for the equivalent N-acetylglucosamine content of an A-substance preparation depending upon the experimental conditions selected for the analysis of N-acetylglucosamine itself and for that of the specific substance preparations. However, the values determined for different A-substance preparations under standardized conditions are highly significant in a relative rather than an absolute way, since the existence of correlations with the serological tests can be demonstrated.

TABLE I  
*Properties of Some Blood Group A-Specific Substance Preparations*

Starting material	Procedure followed in isolation	Note containing description of fraction	Fraction number	Yield (from starting material) Per cent	Inhibition of hemolysis titers <sup>a, bbb</sup>	Inhibition of isoelectrofocusing <sup>a, bbb</sup>	Color with modified Ehrlich reagent <sup>c, dde</sup>
Hog gastric mucin granules, Wilson Lab., Item No. 443	Sodium sulfate fractionation of a centrifuged aqueous suspension (2).	<i>d</i>	R.1F.2A	10	0.075 ± 0.005	45 ± 15	11.7 ± 0.3
			R.2F.2A	15	0.11 ± 0.01	45 ± 15	12.1 ± 0.3
			R.3F.2		0.090 ± 0.010	60 ± 20	12.1 ± 0.3
	Electrodialysis of uncentrifuged aqueous suspension followed by sodium sulfate fractionation.	<i>e</i>					
	Aqueous suspension centrifuged once in Sharples at pH 4.8. Electrodialyzed. Fractionated with ethanol.	<i>f</i> <i>g</i> <i>h</i> <i>i</i>	62	75	0.23 ± 0.01	40 ± 10	7.1 ± 0.4
			111	33	0.19 ± 0.01	75 ± 30	8.4 ± 0.3
			112	26	0.24 ± 0.00	50 ± 15	7.5 ± 0.3
			87	11	0.093 ± 0.005	95 ± 30	11.8 ± 0.3
	Aqueous suspension fractionated with ethanol after preliminary heating at pH 4.2 (3).	<i>j</i>	R.4F.3	25	0.12 ± 0.01	80 ± 20	11.0 ± 0.3
			R.5F.3	29	0.11 ± 0.01	85 ± 20	11.0 ± 0.2
	Aqueous suspension fractionated with ethanol after centrifuging twice at pH 4.4 in Sharples.	<i>k</i> <i>l</i> <i>m</i>	125	60	0.18 ± 0.01	45 ± 15	9.0 ± 0.2
			123	34	0.24 ± 0.01	40 ± 15	8.5 ± 0.3
			128	21	0.11 ± 0.00	75 ± 20	11.8 ± 0.1
		<i>n</i> <i>o</i> <i>p</i>	135	72	0.18 ± 0.02	45 ± 15	7.6 ± 0.2
			142	38	0.24 ± 0.00	45 ± 15	8.1 ± 0.2
			143	22	0.11 ± 0.01	85 ± 20	10.6 ± 0.1

TABLE I—Continued  
*Properties of Some Blood Group A-Specific Substance Preparations*

Starting material	Procedure followed in isolation	Note containing description of fraction	Fraction number	Yield (from starting material) <i>Per cent</i>	Inhibition of hemolysis titer <sup>a,Abb</sup>	Inhibition of isoelectrolytic titration titer <sup>a,Abb</sup>	Color with modified Ehrlich reagent <sup>a,Abb</sup>
	Aqueous suspension fractionated with ethanol after centrifuging at pH 4.4 and then at pH 7.2 in Sharples.	<i>q</i>	97 124	65 51	0.18 ±0.01 0.17 ±0.01	45±15 40±15	9.0±0.1 9.3±0.2
		<i>r</i>	109 115	30 33	0.25 ±0.01 0.24 ±0.01	40±10 45±15	11.0±0.2 11.1±0.1
		<i>s</i>	120 121	15 17	0.10 ±0.00 0.10 ±0.00	75±20 80±20	9.3±0.1 9.6±0.2
		<i>t</i>	127 129	53 51	0.17 ±0.00 0.18 ±0.00	45±15 45±15	10.6±0.1 10.6
		<i>u</i> <i>v</i> <i>w</i> <i>x</i>	39 52 31 36	49 32 13 10	0.20 ±0.04 0.13 ±0.01 0.11 ±0.00 <i>v</i> , Note <i>uu</i>	40±15 50±15 55±10 55±15 <sup>uu</sup>	11.2±0.2 11.4±0.1 11.4±0.1 9.5±0.5 <sup>uu</sup>
	Ethyl alcohol fractionation of aqueous solution of fraction 52.	<i>y</i> <i>z</i>	66 68	4 5	0.080±0.010 0.11 ±0.00	110±30 90±30	12.7±0.1 12.7±0.1
		<i>aa</i> <i>bb</i>	69 71	1 22	0.10 ±0.01 0.12 ±0.01	75±20 80±20	10.7±0.1 12.7±0.1
	Sodium sulfate fractionation of aqueous solution of fraction 52.						

TABLE I—Continued  
Properties of Some Blood Group A-Specific Substance Preparations

Starting material	Procedure followed in isolation	Note concerning description of fraction	Fraction number	Yield (from starting material)	Inhibition of hemolysis titer <sup>a, b</sup>	Inhibition of isoelectroagglutination titer <sup>a, b</sup>	Color with modified Ehrlich reagent <sup>c, d</sup>
Hog Gastric Mucin(4) <sup>4, 5</sup>	Ethyl alcohol fractionation of 90% phenol solution (2, 4). <sup>4</sup>	<i>cc</i>	Kabat 1A(4) 960-GM-2 <sup>4</sup>	<i>Per cent</i>	0.10 ±0.01 0.14 ±0.01	50±20 55±25	11.3±0.1 10.0±0.2
	Tryptic digestion followed by alcohol fractionation <sup>4, 5</sup>	<i>dd</i>	960-GM-1B <sup>4</sup> 960-GM-1C <sup>5</sup>		0.17 ±0.02 0.13 ±0.01	55±25 55±25	9.6±0.2 10.5±0.1
Hog Stomach Linings <sup>6</sup>	Autolysis, followed by alcohol fractionation. <sup>5</sup>	<i>ee</i>	M-330 <sup>6</sup>		0.14 ±0.01	55±25	10.7±0.1
Hog gastric mucin granules. Wilson Lab. Item No. 443	Separation of "neutral polysaccharide" by selective adsorption and precipitation (5).	<i>ff</i>	47	2	0.065±0.010	30±10	13.3±0.5
	Fractionation of part of the product by precipitation with sodium sulfate.	<i>gg</i>	48	5	0.080±0.020	30±10	12.3±0.1

<sup>4</sup> We are indebted to Dr. E. Brand for samples of Kabat's preparation, 1A, and for two Sharp and Dohme preparations, 960-GM-1B and 960-GM-2, as well as for information concerning the methods of preparation of the latter two materials.

<sup>5</sup> We are indebted to Dr. R. H. Barnes, of Sharpe and Dohme, Inc., for a sample of preparation 960-GM-1C and for information concerning the method used in its preparation.

<sup>6</sup> We are indebted to Dr. J. A. Leigity, of the Lilly Research Laboratories, for a sample of preparation M-330 and for information concerning the method used in its preparation.

TABLE I—Continued  
*Properties of Some Blood Group A-Specific Substance Preparations*

Starting material	Procedure followed in isolation	Note containing description of fraction	Fraction number	Yield (from starting material)	Inhibition of hemolysis titer <sup>a,Abb</sup>	Inhibition of isoelectroton titer <sup>a,Abb</sup>	Color with modified Ehrlich reagent <sup>a,Abb</sup>
Pepsin, soluble powder, 1:10000, Wilson Lab.	Digestion of fraction R.4F.3 with papain-HCN. Subsequent fractionation with ethanol and with acetic acid and acetone (3).	<i>hh</i>	R.6F.5A	Per cent 31 <sup>99</sup>	0.10 ±0.01	25±10	12.3±0.2
	Heating fraction R.5F.3 with formamide. Subsequent fractionation with ethanol, acetic acid and acetone(3).	<i>ii</i>	R.7F.5A	26 <sup>uno</sup>	0.050±0.005	10± 5	13.4±0.3
	Alcohol fractionation of an aqueous solution previously heated at pH 6 and pH 3.5 (6).	<i>jj</i>	22	1	0.050±0.005	70±15	11.6±0.3
Fluid from pseudomucinous ovarian cyst from patient of blood group A. <sup>7</sup>	Autolysis, followed by alcohol fractionation (3).	<i>kk</i>	26	2	0.040±0.005	35±10	11.7±0.1
	Centrifugation, followed by alcohol fractionation.	<i>ll</i>	162		0.65 ±0.20	4.5±1.5	1.7±0.3
		<i>mm</i>	113		1.9 ±0.3	4± 3	0.3±0.2
		<i>nn</i>	119		0.27 ±0.03	7± 5	3.4±0.3

<sup>7</sup> We are indebted to Dr. Roy W. Hammack, of the Pathology Laboratories of the Hospital of the Good Samaritan, Los Angeles, Cal., for a sample of fluid aspirated from a pseudomucinous ovarian cyst (No. B-3731-45) removed from a patient of blood group A.



TABLE I—Continued  
 Properties of Some Blood Group A-Specific Substance Preparations

Starting material	Procedure followed in isolation	Note containing description of fraction	Fraction number	Yield (from starting material)	Inhibition of hemolysis titer <sup>a,bb</sup>	Inhibition of isoaagglutination titer <sup>a,bb</sup>	Color with modified Ehrlich reagent <sup>a,bb</sup>
Lyophilized erythrocytes, pooled from a large number of donors of blood group A. <sup>s</sup>	Successive extractions with ethanol-water solutions of decreasing alcohol content, followed by concentration of extract and precipitation with acetone (7). Subsequent fractionation with ethanol.	oo	1	Per cent 0.3	16±2	0.1±0.04	less than 0.3
		pp	56	7 <sup>±</sup>	13±2	0.9±0.5	less than 0.3
		qq	57	22 <sup>±</sup>	140±15	less than 0.03	less than 0.3
		rr	169	60 <sup>±</sup>	45±5		less than 0.3
Lyophilized stromata from pooled erythrocytes from donors of blood group A. <sup>sw</sup>	Extraction with boiling absolute ethanol (7).  Digestion with crude pancreatin at pH 7.9 at 40°C.	ss	45	7 0.2 <sup>±</sup>	34±3	0.2±0.06 <sup>aaa</sup>	less than 0.3
		tt	161	10 0.3 <sup>±</sup>	29±3		0.5±0.3

<sup>s</sup> We are indebted to the Hyland Laboratories, Los Angeles, Cal., for a sample of lyophilized, pooled erythrocytes removed from human donors of blood group A. The erythrocytes were washed twice with physiological saline prior to lyophilization.

Notes. *a.*  $\gamma$  of test substance present in system in which erythrocytes are 50% hemolyzed. *b.*  $\mu$ l. of serum completely inhibited/ $\gamma$  of test substance. *c.* Expressed as equivalent *per cent* of N-acetylglucosamine in test substance. *d.* Insoluble in 30% sodium sulfate. Precipitate electrodialed. Material from supernatant. *e.* Insoluble when supernatant after electro dialysis made 30% in sodium sulfate. Dialyzed. *f.* Material from undialyzed centrifugate. *g.* Material from supernatant of electro dialyzed centrifugate. *h.* Material precipitated upon electro dialysis of centrifugate. *i.* Material soluble when supernatant after electro dialysis made 47% (v/v) in ethanol but insoluble when made 65% (v/v) in ethanol. Again electro dialyzed. Material from supernatant. *j.* Material soluble in 40% (v/v) ethanol but insoluble in 65% (v/v) ethanol. Not dialyzed. *k.* Material from dialyzed centrifugate. *l.* Material insoluble in 40% (v/v) ethanol. Dialyzed. *m.* Material soluble in 40% (v/v) ethanol, but insoluble in 65% (v/v) ethanol. Dialyzed. *n.* Material from undialyzed centrifugate. *o.* Material insoluble in 40% (v/v) ethanol. Not dialyzed. *p.* Material soluble in 40% (v/v) ethanol, but insoluble in 65% (v/v) ethanol. Not dialyzed. *q.* Material from dialyzed centrifugate. *r.* Centrifugate. Material soluble in 30% (v/v) ethanol but insoluble in 65% (v/v) ethanol. Upon reprecipitation, insoluble in 45% (v/v) ethanol. Dialyzed. *s.* Centrifugate. Material soluble in 30% (v/v) ethanol but insoluble in 65% (v/v) ethanol. Upon reprecipitation, soluble in 45% (v/v) ethanol but insoluble in 65% (v/v) ethanol. Dialyzed. *t.* Centrifugate. All of material insoluble in 65% (v/v) ethanol. Dialyzed. *u.* Material insoluble in 10% (v/v) ethanol. Not dialyzed. *v.* Material insoluble in 10% (v/v) ethanol. Dialyzed. *w.* Material from supernatant after electro dialysis of fraction 39. *x.* Material precipitated by electro dialysis of fraction 39. *y.* Material soluble in 47% (v/v) ethanol but insoluble in 65% (v/v) ethanol. Electro dialyzed. Material from supernatant. *z.* As in *y*, except material precipitated upon electro dialysis. *aa.* Precipitated by 30% sodium sulfate. Electro dialyzed. Material from supernatant. *bb.* As in *aa*, except material precipitated upon electro dialysis. *cc.* Preparation made by Sharp and Dohme. *dd.* Preparation made by Sharp and Dohme. *ee.* Preparation made by Lilly Research Laboratories. *ff.* Material not precipitated by  $\text{Zn}(\text{OH})_2$  at pH 7.1 but precipitated by acetone-acetic acid. Material from supernatant after electro dialysis of final precipitate. *gg.* Precipitated by basic lead acetate, then by 75% (v/v) ethanol, then by 94% (v/v) acetic acid. Not subjected to precipitation by  $\text{Zn}(\text{OH})_2$ . Instead, extracted by water. Material insoluble when extract made 30% in sodium sulfate. Electro dialyzed. *hh.* Insoluble in 65% (v/v) ethanol after digestion and dialysis. Then insoluble in 75% (v/v) ethanol containing HCl. Soluble in 90% (v/v) acetic acid, but insoluble upon addition of acetone. Dialyzed. *ii.* Insoluble in 66% (v/v) ethanol in formamide. Precipitated twice by 68% (v/v) ethanol from water and precipitated from 50% acetone-40% acetic acid. Precipitated by 80% (v/v) ethanol from HCl solution. Dialyzed. Precipitated by 90% acetone. Dialyzed. *jj.* Material insoluble in 65% (v/v) ethanol. Reprecipitated. Dialyzed. *kk.* Precipitated by 68% (v/v) ethanol twice, then by acetone-acetic acid, then by ethanol. *ll.* Total solids from the centrifuged fluid. *mm.* Material insoluble when fluid made 1% in sodium acetate and 50% (v/v) in ethanol. Precipitate dialyzed and the non-dialyzable suspension filtered. Filtrate lyophilized. *nn.* Material soluble when fluid made 50% (v/v) in ethanol but insoluble at 66% (v/v) ethanol. Precipitate

taken up in water, filtered through Seitz pad and reprecipitated by 66% (v/v) ethanol. Precipitate taken up in water, filtered, filtrate dialyzed. Solution filtered through Seitz pad. Heavily opalescent filtrate lyophilized. *oo*. Material insoluble in a boiling absolute ethanol extract but soluble in an approximately 50% ethanol extract. Extract concentrated at room temperature, filtered through a Seitz pad. Fraction 1 precipitated by 5 volumes of acetone from a solution 0.9% in NaCl. Not dialyzed. *pp*. Material (from fraction 1) which is insoluble in absolute ether and in absolute ethanol under reflux but which is soluble in water at room temperature. Fraction 56 precipitated when aqueous extract made 50% (v/v) in ethanol. Not dialyzed. *qq*. Like fraction 56 except material soluble when extract made 50% (v/v) in ethanol, but insoluble at 66% (v/v) ethanol. Not dialyzed. *rr*. Like fraction 57 except material soluble when extract made 66% (v/v) ethanol. Not dialyzed. *ss*. Material extracted by boiling absolute ethanol. Extract concentrated under reduced pressure at 35°C. and the residue lyophilized. *tt*. Material soluble and non-dialyzable after digestion for 42 hours and inactivation at 85°C. for approximately 30 minutes. *uu*. Analytical data in doubt because of slight solubility of the fraction in physiological salt solution. *vv*. Yield calculated from fraction R.4 F.3. *ww*. Yield calculated from fraction R.5 F.3. *xx*. Yield calculated from fraction 1. *yy*. Stromata obtained by centrifugation at 25,000 r.p.m. in the Sharples of aqueous suspensions of erythrocytes and retention of the precipitated material. Precipitate washed until essentially free of hemoglobin, centrifuging it at 5000 r.p.m. after each washing. *zz*. Yield calculated from lyophilized erythrocytes. *aaa*. Serological activity of a suspension prepared by dilution of an ethanol solution with physiological salt solution. *bbb*. The analytical data reported are the mean results of duplicate or triplicate analyses and are given together with the average deviation.

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(e.g., fractions 66, 68, 87, R.3 F.2) which are more active than those obtainable by the use of the hitherto described procedures (2, 3) can be isolated by relatively complex, yet still mild, techniques. These more active, undegraded preparations have perhaps a 20% higher A-substance content than those (fractions R.5 F.3, 128, 143, 31) isolated by the less involved procedures, as indicated by the three criteria of activity discussed in the "Experimental" part above.

Kabat and co-workers have reported (21, 22) that the A-substances isolated from commercial hog gastric mucin by the 90% phenol extraction procedure of Morgan and King (2) and by subsequent purifications are about 60% pure on the basis of specifically precipitable hexosamine. Their report is based on the comparative results of a quantitative precipitin test (4) in which A-substance preparations derived only from serologically active hog gastric mucosae were found to participate to the extent of 100% of the hexosamine present. Our experiments indicate that obtaining substances more active than fractions 128 (alcohol fractionation out of water), R.2 F.2A (sodium

sulfate fractionation out of water), and 31 (phenol extraction with subsequent alcohol fractionation) is not unique to the use of hog gastric mucosae from "secretor" hogs. By the procedures outlined in Table I significant amounts of such more active materials can be obtained from Wilson hog gastric mucin also. We do not believe that our findings with respect to the possibility of isolating highly active fractions from commercial hog mucin are in any way at variance with the findings of Kabat and co-workers (21, 22) that some hog gastric mucosae contain A-substance while others do not, and that in the latter mucosae there is material which is superficially similar to A-substance, but which has no significant serological activity. Thus, although the commercial hog gastric mucin with which we and others have worked may contain such material, the fact that no detailed information is available about the structure of either material leaves entirely open the question of what techniques may suffice to separate them.

In much of our preparative work we have used the technique of electrodialysis and have observed certain phenomena which we believe are significant. When an A-substance preparation is electrodialyzed under a potential gradient of *ca.* 18 volts/cm., a solid separates in the cell. This precipitation seems to be associated with an increase in the hydrogen ion activity of the cell and, ultimately, a pH of 4 or less may be attained. Accumulation of the precipitated solid begins near the cellophane membrane separating the central compartment from the anode compartment. The material which is precipitated can be dispersed or dissolved by the addition of alkali. In addition to the A-substance which is present in the precipitate there is a considerable amount dissolved in the clear or only slightly opalescent portion of the solution which overlays the turbid phase. Where comparison has been made between the serological activity of a precipitate obtained by electrodialysis and that of the material dissolved in its supernatant, it has been found that the more soluble fraction is 20-25% more potent in inhibiting hemolysis than the less soluble fraction, in the range where both activities are low (*cf.* fractions 111 and 112) and also where both are high (*cf.* fractions 66 and 68 and fractions 69 and 71). A-substance preparations which give rise in part to precipitated material when they are electrodialyzed cannot be regarded as homogeneous.

The so-called "neutral polysaccharide" (fraction 47), obtained by the procedure of Meyer, Smyth and Palmer (5), in which the original

mucin is heated with 2% sodium carbonate for 15 minutes at 70°C. and from which an "acidic polysaccharide" is removed in acetic acid solution as a gelatin salt, has a very high potency in the inhibition of hemolysis test (see Table I), in accord with the findings of Landsteiner as reported by Meyer *et al.* (5). Its activity in this test is greater than that of any other fraction obtained by us from hog gastric mucin with the single exception of R.7 F.5A which was obtained by heating another A-substance preparation in formamide solution at 150°C. (3). The equivalent N-acetylglucosamine content of fraction 47 is also higher than that of any fraction except R.7 F.5A. We have found, however, that the potency of fraction 47 and the closely related fraction 48 in the inhibition of isoagglutination test is very low, and, thus, is in no way commensurate with the hemolysis inhibition activity. This indication of alteration in A-substance in the course of Meyer's procedure (5) has not previously been recognized, although the findings of Morgan and King (2) with respect to the lability of A-substance in alkaline solution strongly suggest that the initial alkaline treatment used by Meyer *et al.* might profoundly alter the serological properties of the product.

The relative serological activities of fractions R.6 F.5A and R.7 F.5A compared with those of the fractions from which they were derived (see Table I) confirm the findings of Landsteiner and Harte (3) and of Morgan and King (2) with respect to the effects of treating A-substance with papain-HCN or with formamide at 150°C. By both of these procedures the activity of the A-substance in inhibiting hemolysis is increased and in inhibiting isoagglutination is decreased.

We have found that the alcohol fractionation procedure of Landsteiner and Harte (3) with some modifications possesses advantages over other procedures for the preliminary concentration of A-substance from commercial (Wilson) hog gastric mucin for the purpose of further purification and investigation, conveniently providing an undegraded preparation which in yield and potency is equivalent or superior to those obtained by other procedures. The desirable modifications in the Landsteiner and Harte procedure are: a, omission of the initial heating at 100°C.; and b, substitution of a thorough centrifugation in the open bowl of the Sharples of the aqueous suspension of mucin at a pH of 4.5. Centrifugation as indicated serves to remove about 15% of the weight of the mucin granules taken. The fraction which is removed at 40%

(v/v) alcohol concentration is obtained in 35% yield and is 40–50% less active in inhibiting hemolysis than a centrifuged suspension of mucin. The most active fraction is obtained in 20% yield from mucin and has essentially twice the inhibition of hemolysis potency of crude mucin.

### SUMMARY

A study has been made of the relative merits of all previously reported procedures and of some new variants of these in isolating blood group A-specific substance from Wilson hog gastric mucin. In this study it has been possible, for the first time, to make direct comparisons of the serological activities of the fractions arising from all of these procedures. No fraction had more than 2–4 times the activity in inhibiting isoagglutination or 2–3 times the activity in inhibiting hemolysis of a centrifuged, undialyzed suspension of the original mucin. By rather complex procedures materials were obtained which had about 20% more activity in inhibiting hemolysis than those derived by any one of the hitherto described techniques.

Observations are reported on the isolation of A-substance from human erythrocytes and ovarian cyst fluids, and from hog pepsin.

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# The Oxidation of Glycerol by *Escherichia freundii*

M. N. Mickelson<sup>1</sup> and F. E. Shideman

From The Departments of Bacteriology and Pharmacology, Medical School,  
University of Michigan, Ann Arbor

Received January 13, 1947

## INTRODUCTION

In the past ten years numerous investigations on the role of phosphorus in the intermediary carbohydrate metabolism of bacteria have appeared.

In 1935 Tikka (1) suggested that the initial steps of glucose breakdown by *Escherichia coli* may follow the same pathway as the Embden-Meyerhof scheme for muscle and yeast. The ability of fresh living cells of *E. coli* to metabolize added  $\alpha$ -glycerophosphate was demonstrated. Endo (2) found  $\alpha$ -glycerophosphoric acid to be a product of hexosediphosphate breakdown by an acetone-dried powder prepared from *E. coli*.

Werkman, Zoellner, Gilman and Reynolds (3) isolated phosphoglyceric acid from the anaerobic breakdown of glucose by *Escherichia freundii*, the organism used in this investigation. It was the first isolation of this intermediate from a bacterial fermentation.

Relatively little information has been published on the mechanism by which glycerol is metabolized by the coliform bacteria. Anaerobic decomposition of glycerol by *E. freundii* has been investigated by Braak (4) and Mickelson and Werkman (5). Since the present study was initiated, a paper appeared by Gunsalus and Umbreit (6) on the oxidation of glycerol by *Streptococcus faecalis*. This report deals with the role of phosphate in the oxidation of glycerol by *E. freundii*.

## METHODS

The cell suspensions used in this work were prepared from a culture of *E. freundii*. The organism was first described and named *Bacterium freundii* by Braak (4) and later formed the type species of the genus *Citrobacter* of Werkman and Gillen (7). In the fifth edition of Bergey's manual (8) it was placed in the genus *Escherichia*. The organism is a typical representative of the citrate-fermenting, methyl red-positive, Voges-Proskauer-negative members of the coliform group.

The bacterial preparations were made as follows: Ten ml. of a broth medium consisting of 1% glycerol, 0.3%  $(\text{NH}_4)_2\text{SO}_4$  and 1%  $\text{K}_2\text{HPO}_4$ , adjusted to pH 6.8 by

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<sup>1</sup> Present Location, Midwest Research Institute, Kansas City, Missouri.



addition of  $\text{H}_2\text{SO}_4$ , were inoculated with the growth from an agar slant of *E. freundii* and incubated at  $37^\circ\text{C}$ . After three successive transfers in this medium, at 24-hour intervals, the entire culture was used to inoculate 500 ml. of the same medium modified by the addition of 0.1% Difco peptone and containing 0.025%  $\text{K}_2\text{HPO}_4$ . Following 24 hours incubation the entire culture was transferred to 5,200 ml. of the same medium to which was added sterile  $\text{NaHCO}_3$  buffer solution containing 6 g. of  $\text{NaHCO}_3$  /300 ml. of solution. The culture was aerated slowly using an alundum sparger. At the end of 24 hours the cells were separated from the medium with a Sharples centrifuge. The yield varied from 6 to 10 g. of cell paste/6 l. of medium.

The cells were treated further as follows: Lyophilized bacteria were prepared by freezing the freshly harvested bacteria in a dry ice-alcohol bath, drying to a fine powder in a high vacuum, then stored at  $5^\circ\text{C}$ .

Several attempts were made to prepare active cell-free extracts from the freshly harvested bacteria by the technique of Wiggert, Silverman, Utter and Werkman (9). Acetone-treated cells were prepared by the procedures of Bernheim (10) or Krampitz (11).

The frozen cells, which have been employed in most of the experiments, were prepared by placing the cell paste, immediately upon harvesting, in a deep freeze unit at  $-50^\circ\text{C}$ . A suspension prepared from the frozen bacteria was washed twice with distilled water, then resuspended in distilled water for use in manometric experiments. Bacteria stored in this manner retained their original enzymic activity, as measured by their ability to oxidize glycerol, over a period of 5 months.

The frozen bacteria had an endogenous oxygen consumption of 3-4 mm.<sup>3</sup>/mg. of dry weight/hour. The inorganic phosphorus was 1.9 $\gamma$  and the total organic phosphorus 15.6 $\gamma$ /mg. dry weight of cells.

The  $\alpha$ -glycerophosphate used was a 50% aqueous solution of the DL- acid manufactured by the Dr. Theodor Schmidt, Chemische Fab. Gorlitz.  $\beta$ -Glycerophosphate was the sodium salt, used as obtained from Eimer and Amend. The glyceraldehyde employed was a DL- mixture.

Oxygen uptake was determined with a Warburg respirometer at  $38^\circ\text{C}$ . in a 0.1 *M* borate buffer, pH 6.8. Respiratory quotients were determined by the direct method of Warburg. About 5 mg. (dry weight) of bacteria were used in each Warburg flask.

Adenosinetriphosphate was prepared by the method of Needham (12). Glycerol was determined as described by Wood and Werkman (13). Phosphate fractions were determined by an adaptation of the Fiske and SubbaRow method (14) described by Umbreit, Burris and Stauffer (15). Inorganic, 7-minute, 180-minute, and total phosphorus were determined on Warburg flask contents when the manometric measurements were finished. Sufficient 50% trichloroacetic acid was added to give a final concentration of 10% and extraction was carried out at  $0^\circ\text{C}$ . for 24 hours.

Cytochrome oxidase was detected by the procedure of Frei, Riedmüller and Almásy (16).

## RESULTS

Lyophilization of the cells markedly decreased their ability to oxidize glycerol as compared with fresh cells, both in the presence and absence

of added phosphate. Addition of adenosinetriphosphate, boiled yeast extract,  $Mn^{++}$ ,  $Mg^{++}$  and  $Fe^{+++}$  did not restore the total activity of the preparation. Successive washing of the lyophilized cells did not decrease their ability to oxidize glycerol in the absence of added phosphate. Juices prepared from the fresh cells were not capable of oxidizing glycerol in the presence or absence of phosphate. Acetone-treated bacteria lost their capacity to oxidize glycerol completely.

The ability of frozen cells of *E. freundii* to oxidize glycerol,  $\alpha$ -glycerophosphate,  $\beta$ -glycerophosphate and glycerol with added inorganic phosphate is shown in Table I. That glycerol is oxidized is demon-

TABLE I  
*Oxidation of Glycerol and its Phosphorylated Derivatives by  
Cells of E. freundii Preserved in the Frozen State*

Substrate 0.02 M, borate buffer 0.1 M, pH 6.8, phosphate 0.025 M.

Substrate	Mm O consumed/mg dry weight of cells in excess of endogenous		Glycerol disappearance in 2 hours $\gamma$ /mg dry weight of cells	Inorganic phosphorus liberated in 2 hours $\gamma$ / mg dry weight of cells
	1 Hour	2 Hours		
Glycerol	41	46	412	—
$\alpha$ -Glycerophosphate	56	87	—	30
$\beta$ -Glycerophosphate	14	—	—	13
Glycerol + $\alpha$ -Glycerophosphate	110	178	—	29
Glycerol + $\beta$ -Glycerophosphate	108	171	—	21
Glycerol + inorganic phosphate	112	182	917	—

strated by the disappearance of glycerol and the increase in oxygen consumption. On the basis of oxygen consumption values,  $\alpha$ -glycerophosphate is oxidized more rapidly and  $\beta$ -glycerophosphate less rapidly than glycerol. Associated with the increased oxygen uptake in the presence of the phosphorylated esters of glycerol is an increased liberation of inorganic phosphate. More inorganic phosphate is released in the case of  $\alpha$ -glycerophosphate. The sum of the oxygen uptake values when glycerol and  $\alpha$ -glycerophosphate or glycerol and  $\beta$ -glycerophosphate are added separately is less than that obtained when the two are added together.

Addition of inorganic phosphate increases the oxygen consumed in the presence of added glycerol and this oxygen uptake is greater than that obtained when either  $\alpha$ - or  $\beta$ -glycerophosphate is used as a substrate. Concomitant with this stimulation of oxygen uptake by addition of inorganic phosphate in the presence of glycerol is an increased utilization of glycerol.

Experiments similar to that presented in Table I were carried out also with freshly harvested cells. Although the endogenous oxygen consumption of the two cell preparations were equivalent, freshly harvested bacteria oxidized glycerol and its phosphorylated esters only one-half to one-third as much as the frozen cells.

Glycerol disappearance and respiratory quotients were determined in the absence and presence of added inorganic phosphate (Table II).

TABLE II

*Effect of Inorganic Phosphate on the Respiratory Quotient and Glycerol Consumption by E. freundii*

Glycerol 0.02 M; phosphate 0.1 M; R. Q. and glycerol consumption measured during a 20 minute interval.

Experiment	R. Q.		Glycerol disappearance $\gamma$ /flask	
	Glycerol	Glycerol + Phosphate	Glycerol	Glycerol + Phosphate
1	0.58	0.34	260	1330
2	0.71	0.088	370	840
3	0.64	0.114	—	—

The values obtained for the respiratory quotient in the absence of added phosphate are less than, but approach, the theoretical of 0.86 for the complete oxidation of glycerol. Addition of inorganic phosphate produces a marked decrease in the respiratory quotient, which is associated with a marked increase in glycerol consumption. In the absence of added phosphate, glycerol oxidation is from 68 to 83% complete, whereas in its presence oxidation ranges from 10 to 40% complete. These findings suggest that phosphate is exerting its effect upon the initial stages of the oxidation of glycerol.

In view of the stimulatory action of inorganic phosphate on glycerol oxidation, experiments were performed to determine the effects of

various concentrations of this ion (Figs. 1 and 2). Along the ordinate in Fig. 1 are plotted changes in the oxygen consumption in the presence of glycerol produced by varying the concentration of inorganic phosphate. A maximum effect is obtained with 0.1 *M* phosphate. Higher and lower concentrations produce less effect and inhibition is obtained at a concentration of 1 *M*. The effect of optimal phosphate ion con-

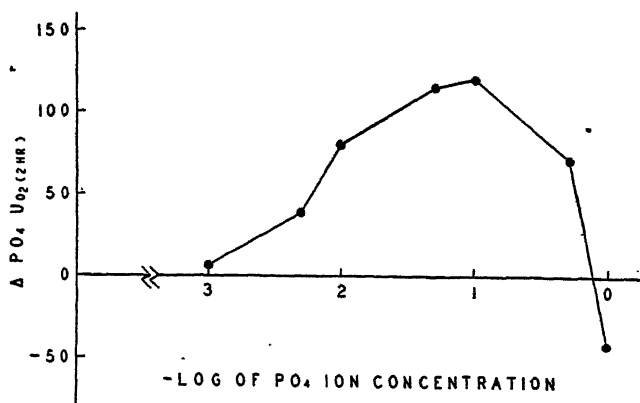


FIG. 1. Optimum concentration of inorganic phosphate for the oxidation of glycerol by *E. freundii*. Phosphate was added as a Sorensen phosphate buffer, pH 6.8.

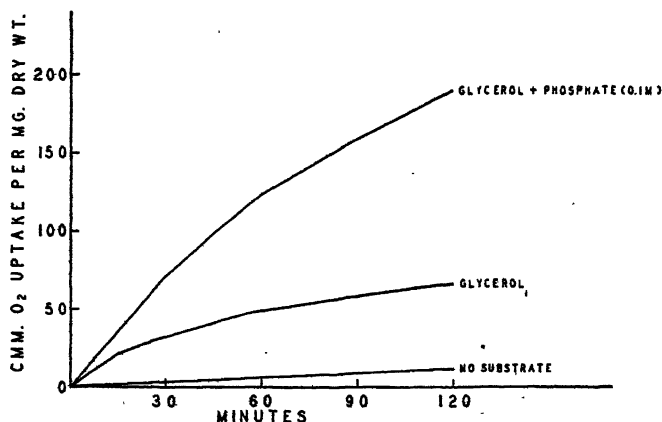


FIG. 2. Effect of inorganic phosphate on the oxidation of glycerol by *E. freundii*. Glycerol 0.02 *M*.

centration on oxygen consumption in the presence of glycerol is presented graphically in Fig. 2.

An uptake in inorganic phosphate when glycerol is oxidized by these organisms is demonstrated in Fig. 3, which shows phosphorylation to be maximum at about 40 minutes. Up to this time oxygen uptake is linear and then drops off rapidly. At the end of 100 minutes, oxygen consumption has dropped to practically zero and most of the inorganic phosphate which has been esterified has reverted to its original form.

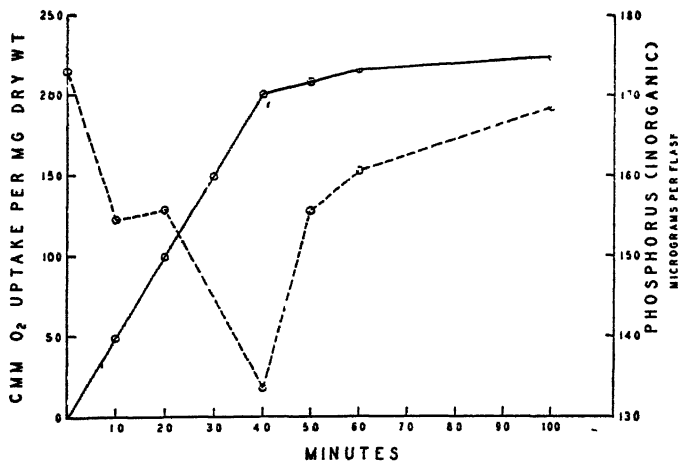


FIG. 3. Oxygen consumption and inorganic phosphate uptake by *E. freundii* in the presence of glycerol and added inorganic phosphate. Glycerol 0.02 *M*; phosphate 0.0025 *M*.

To obtain information on the distribution of the inorganic phosphate which was esterified, experiments were designed to determine changes in the various phosphate fractions during the oxidation of glycerol in the presence and absence of inorganic phosphate. Data from such an experiment are presented in Table III. Addition of inorganic phosphate results in an increase in total organic phosphate, nearly all of which is found in the 180-minute<sup>2</sup> fraction. The possibility that adenosinetriphosphate might function more efficiently as a phosphate donor than inorganic phosphate was tested (Table III). On the basis of oxygen uptake and assuming that one mole of phosphorus per mole of adeno-

<sup>2</sup> Organic P resistant to hydrolysis for 180 minutes in 1 *N* acid.

TABLE III

*Comparative Effectiveness of Adenosinetriphosphate and Inorganic Phosphate for the Phosphorylation and Oxidation of Glycerol by E. freundii*

Glycerol 0.02 M; inorganic phosphate 0.005 M; A.T.P. 0.0017 M.

Substrate	Mm. <sup>3</sup> O <sub>2</sub> consumed/mg. dry weight of cells in excess of endogenous		Increase (+) or decrease (−) in various phosphate fractions in γ P/mg. dry wt./2 hours.			
	1 hour	2 hours	Inorg.	7-min.	180-min. resistant	Total organic
Glycerol	32	37	−0.6	−0.1	+0.3	+0.6
Glycerol + inorganic phosphate	49	66	−1.1	+2.1	+16.0	+18.0
Glycerol + ATP	53	78	+11.0	−7.1	+8.8	−12.0

sinetrisphosphate is available for the phosphorylation, adenosinetrisphosphate appears to be about 2.5 times as efficient as inorganic phosphate. Adenosinetrisphosphate, like inorganic phosphate, produces an increase in that fraction of the organic phosphate which is resistant to 180-minute hydrolysis. In addition, there is a drop in total organic phosphate, an increase in inorganic phosphate and a drop in the 7-minute fraction.

The effects of various inhibitors on the oxidation of glycerol in the absence and presence of phosphate are presented in Table IV. Sodium

TABLE IV

*Effect of Inhibitors on the Oxidation of α-Glycerophosphate and Glycerol in the Presence and Absence of Inorganic Phosphate by E. freundii*

Glycerol 0.02 M; phosphate 0.1 M.

Inhibitor	Concentration of inhibitor	Per cent inhibition with		
		Glycerol	Glycerol + Phosphate	α-Glycerophosphate
Fluoride	0.1 M	68	47	—
	0.075 M	75	3	—
Cyanide	0.01 M	71	88	—
	0.001 M	78	100	—
Iodoacetate	0.0005 M	86	99	85
	0.0001 M	63	92	84

fluoride (0.075 *M*), which is without effect upon oxygen uptake in the presence of phosphate, produces a 75% inhibition of oxygen consumption in the absence of phosphate. Higher concentrations of fluoride (0.1 *M*) inhibit glycerol oxidation in the presence of inorganic phosphate. Iodoacetate (.0001–.0005 *M*) has a somewhat greater inhibitory effect on oxygen utilization in the presence than in the absence of added inorganic phosphate. The inhibitory effect of iodoacetate on oxygen consumption in the presence of  $\alpha$ -glycerophosphate is less than that obtained when glycerol and inorganic phosphate are used as substrate. Cyanide produces an inhibition of oxygen uptake in the absence and presence of added inorganic phosphate.

The presence of cytochrome oxidase in the organism is demonstrated in Table V. The Nadi reagent was prepared according to the method

TABLE V  
*Cytochrome Oxidase Test on Cells of E. freundii*

ABSENCE OF		INTENSITY OF COLOR REACTION			
Glycerol .02 <i>M</i>	KCN .01 <i>M</i>	1 min.	5 min.	10 min.	15 min.
1–	–	+	++	+++	+++
2–	+	–	–	–	–
3+	–	+	++	+++	+++
4+	+	–	–	–	–

of Frei, Riedmüller and Almásy (16). For each experiment 10 mg. (dry weight) of cells were used. In both the absence and presence of glycerol, the color reaction appeared in the course of 1 minute and increased in intensity up to 10 minutes. There was no change in color of the reagent when KCN (0.01 *M*) was present.

The possibility of the oxidation of glycerol to glyceraldehyde prior to phosphorylation was tested (Table VI). Oxygen uptake in the presence of glyceraldehyde is much less than in the presence of glycerol. During the first hour, addition of phosphate produces a marked stimulation of oxygen uptake when glycerol is used as the substrate but not when glyceraldehyde is used. During the second hour there is a slight stimulation of oxygen consumption in the presence of added glyceraldehyde by phosphate but this is of questionable significance.

TABLE VI

*Effect of Inorganic Phosphate on Oxidation of Glycerol  
and Glyceraldehyde by E. freundii*

Glycerol, 0.02 M; DL-glyceraldehyde 0.02 M.

Substrate	Mm. <sup>3</sup> O <sub>2</sub> consumed/mg. dry weight of cells in excess of endogenous	
	1 Hour	2 Hours
Glycerol	50	75
Glycerol + phosphate	97	177
Glyceraldehyde	15	21
Glyceraldehyde + phosphate	15	32

### DISCUSSION

The aerobic metabolism of glycerol by *E. freundii* is similar in several respects to that of *Strep. faecalis* described by Gunsalus and Umbreit (6). Both organisms require phosphate for the oxidation of glycerol. The molar ratio of oxygen consumed to glycerol utilized by *E. freundii* is approximately one, indicating complete oxidation to phosphoglyceric acid. Further evidence for such an overall reaction is the fact that the increase in extractable organic phosphate, occurring during the oxidation of glycerol is found in the 180-minute fraction, which is presumably phosphoglyceric acid. Comparison of oxygen consumption and phosphate uptake of this organism with that of *Strep. faecalis* reveals a marked similarity. During the first 30 or 40 minutes, when oxygen consumption is linear, there is a continuous fall of inorganic phosphate in the medium. As the rate of oxygen uptake falls the amount of inorganic phosphate in the medium increases and reaches the original level in about 100 minutes. Such a finding further suggests an oxidation of glycerol associated with phosphorylation.

With glycerol, and in the absence of inorganic phosphate, the respiratory quotients approach the theoretical value of 0.86 for the complete oxidation of glycerol. With added phosphate there is a marked lowering in the respiratory quotient although more glycerol disappears than in the absence of phosphate. These experiments continued for 20 minutes during which a rapid disappearance of phosphate occurred. Such findings lend further support to an initial phosphoryla-



tive oxidation of glycerol. Whereas, in the presence of inorganic phosphate, the molar ratio of oxygen consumed to glycerol utilized approaches the theoretical value of one for the complete oxidation of glycerol to phosphoglyceric acid, in the absence of added phosphate two moles of oxygen are taken up for every three moles of glycerol consumed. Values for the respiratory quotient in the absence of added phosphate suggest a more complete oxidation of glycerol than in the presence of phosphate, but the ratio of oxygen utilization to glycerol consumption in the former case would indicate a less complete oxidation of glycerol. These paradoxical findings might be explained on the basis that glycerol is metabolized differently when sufficient available phosphate is not present. This organism, unlike *Strep. faecalis*, metabolizes glycerol not only aerobically but likewise anaerobically (5). Under anaerobic conditions, for each two moles of glycerol oxidized to carbon dioxide, acetic acid, formic acid, etc., one mole is reduced to trimethylene glycol. It may be that this organism resorts to an anaerobic mechanism when adequate amounts of phosphate are not available. Such reactions would explain the value obtained for the ratio of oxygen consumed to glycerol disappearing in the absence of added phosphate.

Gunsalus and Umbreit (6) demonstrated the oxidation of the phosphoric acid esters of glycerol by *Strep. faecalis* only when they employed dried cells ground *in vacuo* in the presence of yeast extract. The  $Q_{O_2}$  of such a preparation, when a mixture of  $\alpha$ - and  $\beta$ -glycerophosphates was used as a substrate, did not approach the value obtained with living cells when glycerol was oxidized. As they have suggested, a possible explanation for such a finding is the presence in their preparation of  $\alpha$ - and  $\beta$ -glycerophosphates of the unnaturally occurring D-forms of these esters which inhibit the oxidation of the L- or natural, forms. The organism studied here differs in many respects from *Strep. faecalis* in ability to oxidize glycerol or its phosphorylated esters. The fresh cells of *E. freundii*, or frozen cells thawed for use, are capable of oxidizing  $\alpha$ -glycerophosphate as well as, or better than, glycerol. The  $\alpha$ -glycerophosphoric acid used in these experiments was a mixture of the D- and L- forms. Possibly better oxygen uptake values could have been obtained had a pure preparation of the naturally occurring form been used. The ability of fresh cells to oxidize  $\alpha$ -glycerophosphoric acid is unexplainable if the assumption is made that the living cell is impermeable to phosphate esters. On the other hand, the activity of frozen

cells on the  $\alpha$ -phosphoric acid ester of glycerol could be explained by altered cell permeability caused by freezing and thawing. In contrast to the  $\alpha$ - form,  $\beta$ -glycerophosphoric acid is oxidized only slightly. Such findings suggest that the initial step in the oxidation of glycerol by *E. freundii* is a phosphorylation to  $\alpha$ -glycerophosphoric acid. The more than additive effect on oxygen consumption when glycerol and  $\beta$ -glycerophosphoric acid together are used may result from inorganic phosphate being made available by hydrolysis of phosphoric acid from  $\beta$ -glycerophosphoric acid.

The inhibition of glycerol oxidation by fluoride (.075 *M*) in the absence of added phosphate and the lack of an inhibitory effect in the presence of added phosphate, suggest that the major portion of oxygen consumed when inorganic phosphate is available is utilized in the steps leading to the formation of phosphoglyceric acid (17). A higher concentration of fluoride (0.1 *M*) produces an inhibition of glycerol oxidation in the presence as well as in the absence of added phosphate. This may be due to an inhibition of  $\alpha$ -glycerophosphoric acid dehydrogenase (18) as well as an inhibition of phosphoglyceric acid breakdown. The effect may also result from the formation the stable fluorophosphate thus preventing it from functioning in glycerol oxidation. It is noteworthy that glycerol oxidation in the presence of 0.1 *M* phosphate was inhibited where the level of fluoride was increased. Since iodoacetate inhibits both  $\alpha$ -glycerophosphate and triosephosphate oxidation (19) one could expect an inhibition of glycerol oxidation not only in the absence, but likewise in the presence, of added phosphate.

Linkage of the initial stages of glycerol oxidation to the cytochrome system is indicated by the following facts: (1) in the presence of phosphate, R.Q. measurements indicate glycerol oxidation to be only 10–40% complete, though there is a marked increase in glycerol consumption over the controls where no phosphate is added and in which the R.Q. indicates glycerol oxidation to be 68–83% complete (Table II). (2) R.Q. measurements were made during the first 20 minutes, when esterification of inorganic phosphate is at a maximum (Fig. III). (3) Oxygen uptake was linear with time during the period when R.Q. measurements were made. (4) Cyanide gives nearly complete inhibition of oxygen uptake during this period.

The possibility that glycerol might be oxidized to glyceraldehyde prior to phosphorylation was investigated. Such does not seem to be the

case since glyceraldehyde, as compared with glycerol, is oxidized only very slowly and its oxidation is not stimulated by addition of inorganic phosphate.

### SUMMARY

Glycerol and  $\alpha$ -glycerophosphate are oxidized rapidly by *E. freundii*.  $\beta$ -glycerophosphate is oxidized only at a very slow rate. Inorganic phosphate and adenosinetriphosphate increase the rate of oxidation of glycerol. Adenosinetriphosphate is more efficient than inorganic phosphate in increasing the rate of oxidation of glycerol. The pathway for glycerol oxidation appears to be: Glycerol  $\rightarrow$   $\alpha$ -glycerophosphate  $\rightarrow$  triosephosphate  $\rightarrow$  phosphoglyceric acid. Phosphate is a limiting factor in this series of reactions. The initial stages of the oxidation of glycerol are linked to the cytochrome system.

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## The Effect of Insulin on the Concentration of Diphosphothiamine in the Blood\*

Piero P. Foà, Jay A. Smith and Harriet R. Weinstein

*From the Department of Physiology and Pharmacology of  
The Chicago Medical School †*

Received February 26, 1947

### INTRODUCTION

The available evidence on the mechanism of action of diphosphothiamine indicates that it is a catalyst for the oxidation of pyruvic acid and can thus enhance the utilization and storage of carbohydrates (1). In this respect diphosphothiamine works toward the same end as insulin. However, the question of the influence of thiamine on the concentration of glucose in the blood, and on the response of blood sugar to insulin, is as controversial as is the place of thiamine in the therapy of diabetes (2).

It has been reported that thiamine increases the patient's alkaline reserve (3) and carbohydrate tolerance, but the evidence is contradictory (4-8). Diabetic neuritis is similar in many respects to neuritis due to thiamine deficiency. It occurs in patients consuming grossly inadequate diets or at the beginning of insulin therapy, when the vitamin:calories ratio is decreased by an increased carbohydrate intake and utilization (9). On this basis, thiamine alone, or in combination with other B vitamins, has been widely used in the treatment of diabetic neuritis. The results have been doubtful in some instances (10, 11) and good in others (12), especially when thiamine treatment was associated with a good control of the diabetic condition. Despite the fact that thiamine excretion was found to be normal in most diabetic patients (11, 14, 15) and that experimental pancreatic diabetes does not seem to alter the rate of disappearance of injected pyruvate (16) nor the time required to develop thiamine deficiency (17), the possibility exists that thiamine metabolism in diabetes might be impaired. The "normal" urinary excretion of thiamine after a test dose could be due to the fact that the vitamin is being eliminated without being utilized, even in the presence of low storage. Preliminary studies seem to show that the urinary excretion of thiamine in the diabetic is greater following a test dose of thiamine hydrochloride

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\* This work was made possible by a grant from the John and Mary R. Markle Foundation.

† 710 S. Wolcott Avenue.

then when an equivalent amount of thiamine diphosphate is injected (18); this would indicate that the latter might be utilized more efficiently.

The literature on the mechanism of action of insulin has been recently reviewed (19-22). The evidence at hand is consistent with the theory that insulin promotes the conversion of glucose to some intermediate substance necessary for both utilization and storage of carbohydrate. This substance is probably formed during the initial stages of glucose metabolism (23, 24) and may possibly be glucose-6-phosphate or an "activated" glucose readily transformed into this ester (20). The catalytic action of insulin on the phosphorylation of glucose may be an indirect one and be mediated in part by hexokinase (25, 26).

The present work was done to test the working hypothesis that insulin catalyzes the phosphorylation of thiamine, and that, therefore, in the absence of optimal amounts of insulin the vitamin cannot be converted as readily into its active form.

## METHOD

The experiments were performed on 17 unanesthetized normal dogs and repeated on 6 of them after pancreatectomy. Thiamine hydrochloride was dissolved in saline at the concentration of 1 mg./cc. and was injected intravenously in doses of 1 mg./kg. Insulin was injected subcutaneously in doses of 1 I.U./kg. Samples of blood from the crural vein were obtained before the injection of insulin or thiamine and at various intervals of time afterward. Six normal dogs received both insulin and thiamine. The total amount of blood withdrawn was about 50 cc. Analyses for glucose (27), inorganic phosphate (28), thiamine and diphosphothiamine (29, 30) were begun immediately.

The samples obtained after thiamine injection were treated as follows: 1 cc. of blood was delivered with shaking into a 15 cc. centrifuge tube containing 4 cc. of 1% acetic acid and the tube immersed in boiling water for 10 minutes. After cooling, the samples in which diphosphothiamine was to be determined were incubated for 90 minutes at 45-50°C. with 1 cc. of 3% takadiastase in 0.6 M sodium acetate (the takadiastase solution was freed of any fluorescent material by absorption with Decalso). The samples in which thiamine was to be determined were incubated with sodium acetate alone. After incubation and cooling, the volume was adjusted to 10 cc. and centrifuged at high speed for 30 minutes or more. Five cc. of the supernatant fluid were diluted to 25 and allowed to drip through a 6 cm. column of washed Decalso. The column was then washed 3 times with cold distilled water and then eluted with acid potassium chloride solution (0.8 cc. conc. HCl in 100 cc. 25% KCl). The speed of drop was about 1 cc./min. and the volume of the eluate was 25 cc. The samples of blood obtained when no thiamine was injected were treated as described above, except that 5 cc. were delivered into 20 cc. of 1% acetic acid, 5 cc. of takadiastase solution was used, the volume adjusted to 50 cc. and 40 cc. of supernatant liquid were absorbed. Five cc. of eluate were oxidized in the Hennessy funnel by shaking for 1.5 minutes with 3 cc. of alkaline ferricyanide (3 cc. of 1% potassium ferricyanide diluted to 100 cc. with 15% NaOH) and 18 cc. of redistilled isobutanol.

The funnel was then centrifuged for about 1 minute at low speed, the aqueous layer discarded and the isobutanol layer dried with sodium sulfate, recentrifuged and poured into the fluorimeter cell. A Lumetron fluorimeter was used and adjusted to read 0 with the reagent blank and 100 with a standard solution of thiamine. The fluorimeter was checked with a solution of quinine sulfate. Two determination blanks with and without takadiastase were also prepared; their fluorescence was usually negligible and when greater than 1% of the standard all determinations were discarded.

Recoveries of thiamine and diphosphothiamine added to control samples of blood varied between 85 and 105%.

The statistical significance of all points of each curve was computed according to the method described by Fisher (31) using all determinations made. The probability of the difference between the means being due to chance is given in the caption of each figure.

Depancreatized dogs were used after the operative wound had completely healed (about 10 days) and after insulin had been withheld for 48 hours or until marked hyperglycemia and glycosuria had developed. The same dogs were again used after they had been controlled with insulin for one week or more.

Two control experiments were performed on dogs receiving no injections.

## RESULTS

The results of all experiments of a given type were qualitatively and quantitatively similar and, therefore, the average values have been used in the preparation of the graphs shown in Figs. 1-5.

Fig. 1 shows that the intravenous injection of thiamine hydrochloride into a normal dog is followed by an immediate rise in the concentration of free thiamine in the blood. The concentration reaches an average value of 272  $\gamma$ /100 cc. of blood and gradually decreases toward the initial level. The figure further shows that the concentration of thiamine diphosphate also rises, reaching an average maximum of 55  $\gamma$ /100 cc. (a rise of 458%) in 1-3 minutes and gradually declines toward the initial level during the 15 minute experimental period. The blood sugar remains practically constant while the blood inorganic phosphate decreases approximately 0.7 mg./100 cc., or 19%, during the first minute of the experiment. This drop coincides with the peak of the diphosphothiamine curve and, although not very pronounced, it appeared in all experiments and is statistically significant.

Fig. 2 shows that the injection of insulin into a normal dog is followed by a drop in the concentration of glucose to an average value of 48 mg./100 cc. The inorganic phosphate falls to an average value of 2.4 mg./

100 cc., a decrease of 32.5%. The concentration of free thiamine in the blood does not vary significantly, whereas that of diphosphothiamine rises from an average value of 7.5  $\gamma$ /100 cc. to an average value of 15.5  $\gamma$ /100 cc., or 115%. As can be seen, the rise in the concentration of diphosphothiamine and the drop in the concentration of inorganic phosphate last for at least 4 hours, at which time the action of insulin, as indicated by the hypoglycemia, still persists.

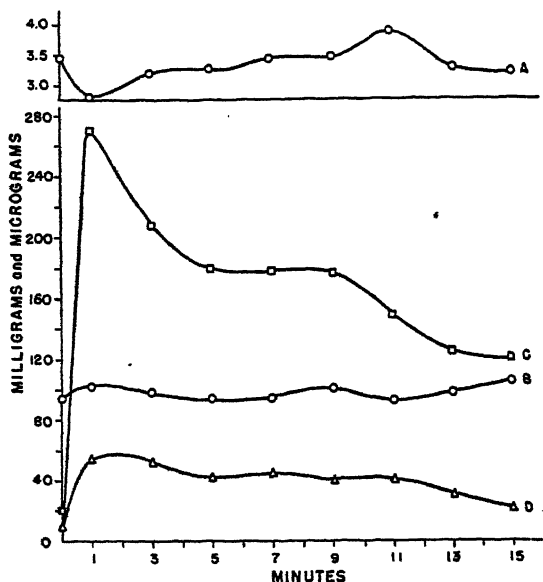


FIG. 1. Eight normal dogs receiving 1mg. of thiamine hydrochloride/kg. of body weight intravenously. Average of 8 experiments. The probability of the decrease in inorganic phosphate and the increase in thiamine and diphosphothiamine being due to chance is less than 1%. The changes in glucose concentration are not significant.

A = Inorganic Phosphate in milligrams; B = glucose in milligrams; C = Free Thiamine in micrograms; D = Diphosphothiamine in micrograms. All values represent concentrations per 100 cc. of blood.

Fig. 3 shows that the intravenous injection of thiamine hydrochloride into a normal dog, 1 hour after the administration of insulin, is followed by an immediate rise in the concentration of free thiamine in the blood. The concentration reaches an average value of 311  $\gamma$ /100 cc. of blood and gradually decreases toward the initial level. The initial concentration of diphosphothiamine is higher than in the normal dogs

receiving no insulin and reaches a maximum value of 103  $\gamma$ /100 cc. of blood, corresponding to a rise of 880%. This rise is greater than that which occurs in normal dogs receiving no insulin. The concentration of blood inorganic phosphate decreases 22% to a minimum value of 1.65 mg./100 cc. and remains low throughout the 15 minute experimental period. The blood sugar remains practically constant at the hypoglycemic level due to the injection of insulin.

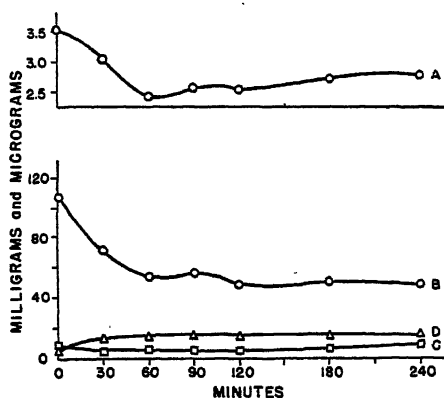


FIG. 2. Nine normal dogs receiving 1 unit of regular insulin/kg. of body weight subcutaneously. Average of 9 experiments. The probability of the changes in inorganic phosphate, glucose and diphosphothiamine being due to chance is less than 1%. The changes in thiamine concentration are not significant. Letters as in Fig. 1.

Fig. 4 shows that the injection of thiamine hydrochloride into depancreatized dogs receiving no insulin is followed by a rise in the concentration of free thiamine to an average value of 156  $\gamma$ /100 cc. In contrast to the normal dog, however, the diabetic dog shows no significant changes in blood diphosphothiamine and the concentration of blood inorganic phosphate fails to decrease at the beginning of the experiment. The small rise in the concentration of blood inorganic phosphate is of doubtful statistical significance. The concentration of glucose does not vary significantly.

Fig. 5 shows the results obtained when thiamine was injected into the same depancreatized dogs after they had received daily injections of insulin in amounts sufficient to allow the excretion of only traces of sugar. As may be seen, the injection of thiamine is followed by a



persistent rise in the concentration of diphosphothiamine to an average value of 40  $\gamma$ /100 cc., or 340%; the glucose curve shows no significant changes; the concentration of free thiamine rises to an average value of 192  $\gamma$ /100 cc. In contrast to the normal dog, the depancreatized controlled dog shows no significant change in the blood inorganic phosphate.

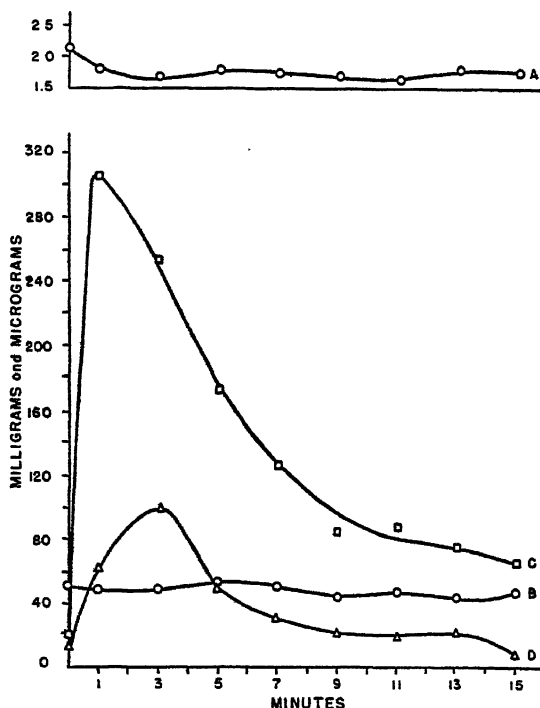


FIG. 3. Six normal dogs receiving 1 mg. of thiamine hydrochloride/kg. of body weight intravenously, one hour after the subcutaneous injection of 1 unit of regular insulin per kg. Average of 6 experiments. The probability of the changes in inorganic phosphate, thiamine and diphosphothiamine being due to chance is less than 1%. The changes in the concentration of glucose are not significant. Letters as in Fig. 1.

Repeated sampling of blood in two dogs receiving no injections did not modify significantly the concentration of thiamine, diphosphothiamine, inorganic phosphate or glucose in the blood.

## DISCUSSION

The rise in the concentration of blood diphosphothiamine following the intravenous injection of thiamine occurs in very few minutes, a phenomenon which had already been observed in man (32). This increase can be interpreted in two possible ways: (a) that preformed diphosphothiamine is liberated from the tissues, or (b) that the injected

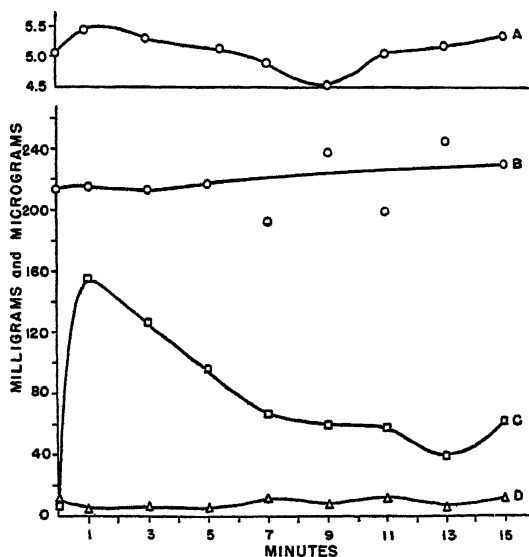


FIG. 4. Six depancreatized hyperglycemic dogs receiving 1 mg. of thiamine hydrochloride/kg. of body weight intravenously. Average of 6 experiments. The probability of the changes in the concentration of thiamine being due to chance is less than 1%. The probability of the rise in phosphate being due to chance is about 6%. The changes in glucose and diphosphothiamine are not significant. Letters as in Fig. 1.

thiamine is phosphorylated. The first interpretation does not seem likely because no rise in diphosphothiamine was observed in the diabetic animals, and especially because a relatively high concentration of thiamine in the blood would probably result in a movement of diphosphothiamine toward the tissues rather than away from them. If the second interpretation is accepted the experimental results would indicate that insulin increases the phosphorylation of thiamine. Our

experiments do not indicate whether this is a direct or an indirect action of insulin.

The meaning of the decrease in the concentration of inorganic phosphate is not clear. Its occurrence at the time when diphosphothiamine rises as well as the fact that it appeared consistently in all experiments on normal animals suggests that inorganic phosphate is utilized in the conversion of thiamine to diphosphothiamine, probably through the mediation of adenosine triphosphate (33). This decrease in phos-

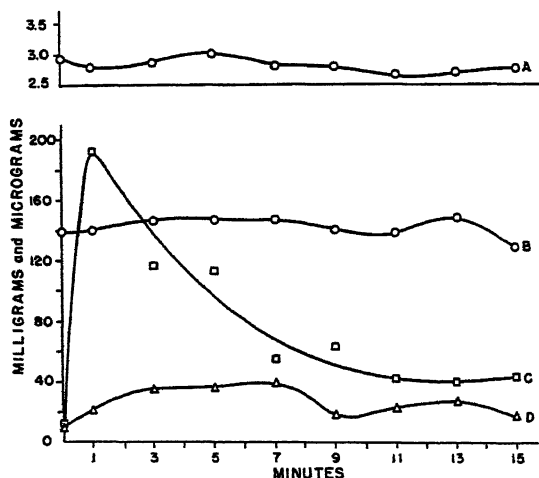


FIG. 5. Six depancreatized dogs controlled with insulin receiving 1 mg. of thiamine hydrochloride/kg. of body weight intravenously. Average of 8 experiments. The probability of the changes in the concentration of thiamine and diphosphothiamine being due to chance is less than 1%. The changes in inorganic phosphate and glucose are not significant. Letters as in Fig. 1.

phate would be analogous to that observed in the normal, but not in the diabetic animal, following the administration of glucose (34). In our experiments there were no significant changes in the concentration of glucose to which the decrease in the concentration of phosphate could be attributed. We do not understand why the concentration of inorganic phosphate did not behave similarly in the depancreatized animals controlled with insulin. The fact that the initial concentration of phosphate in the blood had already been reduced by the insulin does not appear to be the cause of this phenomenon because a decrease

in the concentration of inorganic phosphate occurred in the blood of normal dogs receiving thiamine after insulin.

One must keep in mind that, with the small doses of thiamine used, a great drop in inorganic phosphate could not be expected even if all the injected thiamine were phosphorylated. For this reason an attempt to obtain a more marked decrease in the concentration of inorganic phosphate was made by injecting massive doses of thiamine (20-100 mg./kg.) intravenously. Ten dogs were used. The drop in inorganic phosphate was more pronounced than that in the animals receiving smaller doses of thiamine, but the experiments are of doubtful significance as large doses of thiamine are very toxic and result, among other things, in respiratory paralysis. Although these dogs were kept alive by artificial respiration, a certain degree of anoxia was undoubtedly present. Changes in the blood phosphates due to anoxia have been described (35, 36).

The results can be interpreted to mean that a certain amount of insulin must be present if thiamine is to be phosphorylated. This interpretation is based on (a) the increase in the concentration of diphosphothiamine in the blood following the injection of thiamine or insulin into normal dogs, (b) the greater increase in diphosphothiamine when thiamine is injected after the administration of insulin, (c) the decrease in inorganic phosphate when diphosphothiamine increases, and (d) the failure of the concentration of diphosphothiamine in the blood to increase following the intravenous injection of thiamine into hyperglycemic depancreatized animals. Since thiamine is not biologically active unless converted to its diphosphate, the results may offer an explanation for the appearance of neuritis in diabetes and for the necessity of balancing the patient with insulin if treatment of the neuritis is to be successful. The increased phosphorylation of thiamine brought about by insulin is not surprising in view of the fact that the administration of insulin is followed by an increase in the adenosine-triphosphate of the liver (37) and ATP is the phosphate donor for thiamine (1). It may be recalled that insulin is believed to catalyze the formation of hexosemonophosphate (20), that the energy for this phosphorylation apparently derives from the simultaneous oxidation of pyruvic acid (20) and this, in turn, cannot proceed in the absence of diphosphothiamine (1). These reactions are believed to occur, for the most part, in the tissues. Work is in progress to study the action of insulin on the phosphorylation of thiamine by various tissues *in vitro*.

## ACKNOWLEDGMENTS

Thiamine was generously supplied by the Upjohn Company; Insulin by Eli Lilly and Company.

## SUMMARY

1. Thiamine hydrochloride (1 mg./kg.) injected intravenously into normal dogs is followed by a temporary rise in blood diphosphothiamine associated with a slight but consistent drop in blood inorganic phosphate. No significant changes occur in the concentration of blood sugar.

2. Insulin (1 I.U./kg.) injected into a normal animal is followed by a rise in blood diphosphothiamine.

3. The injection of thiamine hydrochloride (1 mg./kg.) into normal dogs previously treated with insulin (1 I.U./kg.) is followed by changes in the blood concentration of inorganic phosphate and diphosphothiamine which are greater than those occurring in normal dogs receiving no insulin. There are no significant changes in the concentration of blood sugar.

4. The injection of thiamine hydrochloride into depancreatized hyperglycemic dogs is not followed by significant changes in the blood concentration of diphosphothiamine, glucose and inorganic phosphate.

5. Depancreatized dogs controlled with insulin respond like normal dogs to the intravenous injection of thiamine, but do not show a decrease in blood inorganic phosphate.

6. The results are consistent with the hypothesis that insulin increases the phosphorylation of thiamine.

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# The Effect of the Death Rate in Biological Experiments on the Validity of Observations, and on the $\chi^2$ Test for Association

Peter Gray

*From the University of Pittsburgh*

Received March 7, 1947

## INTRODUCTION

The majority of biological experiments are concerned, in a final analysis, with observing the effect of something on a group of living objects. This definition holds, whether we are observing the effect of an inevitable interference, as the effect of time on shape in a growth analysis; or whether we are recording the results of artificial interference, as the operative techniques of the experimental embryologist. At first sight, therefore, it might appear that the results of an experiment could validly be recorded as the number, or proportion, of those which showed the effect in comparison with those which did not; or in recording the degree of the effect in comparison with some existing standard. This is, indeed, the customary procedure in current biological literature where will be found the statement that such-and-such a percentage showed a given effect. It is not always immediately apparent that the percentage referred to can be based on either of two totally different measures: these are the survivors of the experiment, or alternatively, the total number of animals used. These two measures differ from each other as the death rate becomes greater or less. There appears to be no fixed rule in any branch of the biological sciences as to which measure shall be used, nor any realization that the difference between the two measures may affect the validity of the results. The purpose of the first part of this paper is to examine the effect of the death rate on the validity of observations in an isolated experiment: the second part deals with the effect of the death rate on the validity of comparisons between two populations.



# EFFECT OF THE DEATH RATE ON OBSERVATIONS OF A SINGLE POPULATION

Any discussion of this nature can most easily be conducted in symbolic form. Translating into this form the anomaly already mentioned and the assumptions on which it is based, it can be said that any population of  $N$  experimental animals is composed of some ( $x$ ) which do not show a given effect; some ( $y$ ) which show the effect; and some ( $z$ ) which die. Therefore,

$$N = x + y + z \quad (1)$$

The anomaly lies in the fact that some observers state that

$$y\% = \frac{100 y}{x + y} \quad (2)$$

since their results are recorded, either admittedly or by implication, in terms of survivors. Other observers record in terms of the total population, thus stating that

$$y\% = \frac{100 y}{x + y + z} \quad (3)$$

It is obvious that both statements cannot be true unless  $z = 0$ , a most improbable contingency in an experiment involving more than a very few animals. Though there have been discussions as to whether (2) or (3) is the better usage it may readily be shown that either may be incorrect, because the usual experimental observation gives no information as to how many of the dead would have shown the recorded effect had they lived. Translating into symbolic form, let  $y_t$  be the true number affected. Then,

$$y_t = y + kz, \quad (4)$$

where  $kz$  is the proportion of  $z$  which would have shown the effect had they lived. It is further obvious that  $k$  can vary between the limiting values 1 (all the dead would have shown the effect) and 0 (none of the dead would have shown the effect), so that there are two limiting derivatives of (4):

$$y_t = y \quad (y_t = y + kz, k = 0), \quad (5)$$

$$y_t = y + z \quad (y_t = y + kz, k = 1) \quad (6)$$

Before discussing the conditions under which the difference between (5) and (6) can rise to a value which will affect the statistical validity

of the observations, it is interesting to examine one of the possible intermediate values of  $k$ .

Under certain circumstances it can be shown, either by control experiments or by logical deduction, that those factors of the experimental conditions which affect the value of  $y$  cannot have any effect on the value of  $z$ . Or, more simply, that such deaths as occur are not produced by the experimental conditions. When this is true it is logical to suppose that the same proportion of the effect would have been found in the dead group as was found in the survivors. This proportion is defined in identity (4) as  $k$ , so that in this case

$$k = \frac{y}{x + y}. \quad (7)$$

Substituting (7) in (4):

$$y_1 = y + \frac{y}{x + y} z, \quad (8)$$

which may be written in the percentage form as

$$\begin{aligned} y_1\% &= \frac{100 \left[ y + \frac{y}{x + y} z \right]}{x + y + z} \\ &= \frac{100 [y(x + y) + yz]}{(x + y)(x + y + z)} \\ &= \frac{100y}{x + y}. \end{aligned} \quad (9)$$

But (9) is identical with (2), which is the expression used in those cases in which the results are recorded in terms of "percentage of survivors." It can also be shown that (2) cannot be correct unless (7) is also correct, which leads to the general deduction that "results cannot correctly be expressed in terms of 'percentage of survivors' unless it can be shown, either that the death rate in the experiment is not produced by the experimental condition or that the death rate is so small that it may be ignored." This is one of those theorems to which it may be objected that it is a self-evident proposition. Reference, however, to current biological literature will show numerous cases in which results are recorded in terms of percentages of survivors without any reference to the death rate.

Returning now to a consideration of (5) and (6), it is required to

determine under what circumstances  $y$  cannot differ significantly from  $(y + z)$ ; that is, in terms of the theorem given above, under what circumstances the death rate is so small that it may be ignored. "Significant difference" is here used in the statistical sense to indicate a difference which is unlikely to have arisen by chance. If, in the circumstances under discussion, it can be shown that the difference between  $y$  and  $(y + z)$  could have arisen fortuitously, it is evident that either could be used to represent the true value  $y$ . The simplest test of significant difference for figures of this type is the "chi square"\* test of association; as this will be referred to many times in the discussion which follows, it would be well here to explain it in its simplest terms.

Suppose two groups of results. In the first of these  $a$  show an effect and  $b$  do not; in the second  $c$  show the same effect and  $d$  do not. It is desired to find out whether the relative values of  $a$  and  $c$  could have arisen fortuitously; if this is very improbable,  $a$  and  $c$  are considered to be significantly different from each other. The values and relationships of  $a$ ,  $b$ ,  $c$ , and  $d$  may be written down in the rectangular diagram, known as a contingency table, shown below.

$a$	$b$	$a + b$
$c$	$d$	$c + d$
$a + c$	$b + d$	$a + b + c + d$

This diagram is convenient since it permits the value of all possible paired sums, as well as the total summation of,  $a$ ,  $b$ ,  $c$ , and  $d$  to be readily ascertained. The value of " $\chi^2$ " is derived from this table according to the formula:

$$\chi^2 = \frac{(ad - bc)^2(a + b + c + d)}{(a + c)(b + d)(c + d)(a + b)} \quad (10)$$

$P$  (the probability of random distribution) is related to " $\chi^2$ " in such a manner that, as " $\chi^2$ " become greater,  $P$  becomes smaller. The numerical relationships of  $P$  and " $\chi^2$ " have been tabulated and a working convention established that a probability against random distribution of 20 to 1 ( $P = .05$ ) shall be considered the upper limit of possible significance. A value of .05 for  $P$  corresponds to a " $\chi^2$ " value of 3.84;

\* This value will be represented  $\chi^2$  throughout the balance of this paper.

a " $\chi^2$ " value of 4, derived by numerical substitution in a contingency table of the type shown, indicates a probability of about 28 to 1 against the explanation that the values substituted for  $a$  and  $c$  could have been obtained by chance. That is,  $a$  and  $c$  are significantly different. It is thus evident that by substituting  $y$  and  $(y + z)$  for  $a$  and  $c$  in the above table, and then finding under what circumstances " $\chi^2$ " can equal or exceed 4, it is possible to discover the conditions under which they become significantly different from each other; conversely, it can be found under what conditions the difference between them could never become significant, so that either could be employed for  $y$ , without interfering with the validity of the observation.

In erecting the required contingency table it is important to remember that the population under discussion is composed of the total of three factors  $(x + y + z)$ , so that, if only  $y$  show the effect (5),  $(x + z)$  will not show it: correspondingly, if  $(y + z)$  show the effect (6), only  $x$  will not show it. Under these circumstances, the contingency table takes the form:

$x + z$	$y$	$x + y + z$
$x$	$y + z$	$x + y + z$
$2x + z$	$2y + z$	$2(x + y + z)$

so that, from (10),

$$\begin{aligned}
 \chi^2 &= \frac{[(x + z)(y + z) - xy]^2 [2(x + y + z)]}{(2x + z)(2y + z)(x + y + z)^2} \\
 &= \frac{2(xz + yz + z^2)^2}{(2x + z)(2y + z)(x + y + z)} \\
 &= \frac{2z^2(x + y + z)}{(2x + z)(2y + z)} \quad (11)
 \end{aligned}$$

But it has already been stated that  $y$  cannot differ significantly from  $y + z$  unless (11) is equal to, or greater than, 4. Let, therefore,

$$\begin{aligned}
 \frac{2z^2(x + y + z)}{(2x + z)(2y + z)} &\geq 4, \\
 2z^2(x + y + z) &\geq (4y + 2z)(4x + 2z). \quad (12)
 \end{aligned}$$

Substituting (1) in (12):

$$\begin{aligned}
 2Nz^2 &\equiv (2N + 2y - 2x)(2N + 2x - 2y), \\
 z^2 &\equiv \frac{[2N - 2(x - y)][2N + 2(x - y)]}{2N}, \\
 z^2 &\equiv \frac{4N^2 - 4(x - y)^2}{2N}, \\
 z &\equiv \sqrt{2N - \frac{2(x - y)^2}{N}}. \tag{13}
 \end{aligned}$$

It is thus demonstrated that the death rate in an experiment is without significance unless expression (13) be true. But if there is no significant difference between  $y$  and  $(y + z)$ , there is proportionately an even less difference between  $(x + y)$  and  $(x + y + z)$ , so that expression (2) does not differ significantly from expression (3). The theorem given on page 463 can now be completed in this form: "In observing the effect of anything on a biological population, the number affected can only be correctly expressed in terms of 'percentage of survivors' under two conditions. First, when it can be shown that the death rate is not influenced by the experimental conditions. Second, when it can be shown that the number dying is not equal to, nor greater than, the square root of the expression obtained on diminishing twice the total number involved in the experiment by the quotient obtained by dividing twice the square of the difference between those affected and those not affected by the total number involved." This theorem can, of course, be much more easily expressed in symbolic form as: "Given any experimental population  $N$ , in which  $x$  do not show an effect,  $y$  show an effect and  $z$  die, the true percentage of those affected ( $y_i\%$ ) is given by the expression

$$y_i\% = \frac{100(y + kz)}{(x + y + z)}, \tag{14}$$

where  $k$  is the proportion of the dead which would have shown the effect had they lived. This expression may, however, be written

$$y_i\% = \frac{100y}{(x + y)} \tag{15}$$

provided that

$$k = \frac{y}{(x + y)} \quad (7)$$

or that

$$z \cong \sqrt{2N - \frac{2(x - y)^2}{N}}. \quad (16)$$

There remain for discussion only those conditions under which

$$y_t\% = \frac{100y}{(x + y + z)}, \quad (17)$$

which is, as has already been pointed out (3), the form in which results are most usually expressed. It is immediately apparent that there are no circumstances which will justify the use of this expression. For either it is not significantly different from (15), in which case it is redundant: or else it is significantly different from (15), in which case it cannot be equal to (14) and is therefore inaccurate. The extent of inaccuracy is, however, limited: for, from (5) and (6) it is evident that

$$\frac{100y}{(x + y + z)} = y_t\% = \frac{100(y + z)}{(x + y + z)}. \quad (18)$$

But the left hand expression is here " $y\%$ " in terms of the whole so that

$$\begin{aligned} (y_t\%) - (y\%) &= \frac{100(y + z)}{(x + y + z)} - \frac{100y}{(x + y + z)} \\ &= \frac{100z}{(x + y + z)} \end{aligned} \quad (19)$$

and it is apparent that (19) is itself " $z\%$ " in terms of the total population. This conclusion may be presented in the form: "In observing the effect of anything on a biological population, the number affected cannot be accurately expressed as a percentage of the total population if deaths occur. When, however, the number is so expressed it represents an approximation which cannot be too great but which may be too small to the extent of the death rate expressed as a percentage of the total population."

To summarize, then, this first portion of the investigation, it appears that the biologist claiming an effect has to choose between accuracy and an approximation with a variable margin of safety. Accuracy can

be assured only when expression (2) is employed and subjected to the control of expression (13). It would seem to be in the interests of the science that these expressions should be used wherever possible. Where, however, expression (17) must be used, the worker does not run the risk of claiming too great validity for his results, unless the experiment is the very unusual one of endeavoring to show a diminution of effect. In any event it must be strongly recommended that attention be drawn to the possible extent of the error by the publication of the number of deaths, in addition to the number of survivors; this is by no means current practice, particularly in the fields of genetics and embryology.

#### EFFECT OF THE DEATH RATE ON THE COMPARISON OF TWO POPULATIONS

So far this discussion has been concerned solely with the effect of the death rate on the validity of an observation made on a single population. The considerations and formulae here derived do not apply to the comparison of two populations. Reference to expression (11) and to the contingency table immediately above it will show clearly that, though  $y$  may not differ significantly from  $(y + z)$  in a single population, it would not be possible to write one for the other in (11) and obtain a constant value of " $\chi^2$ ." Even if this were possible, it would involve the improbably valid assumption that  $k$  of expression (4) remained constant in two experiments in which the other factors of the expression changed in value. It is therefore only possible to examine the two separate populations  $N (= x + y + z)$  and  $N_1 (= x_1 + y_1 + z_1)$ , in which:

$$y_t = y + kz \quad (k \text{ varying between } 0 \text{ and } 1), \quad (20)$$

$$y_{t1} = y_t + k_1 z_1 \quad (k_1 \text{ varying between } 0 \text{ and } 1). \quad (21)$$

To compare  $y_t$  with  $y_{t1}$  by " $\chi^2$ " it is necessary to erect a contingency table of this form:

$x + (1 - k)z$	$y + kz$	$N$
$x_1 + (1 - k_1)z_1$	$y_1 + k_1 z_1$	$N_1$
$x + x_1 + (1 - k)z + (1 - k_1)z_1$	$y + y_1 + kz + k_1 z_1$	$N + N_1$

For if  $(y + kz)$  show an effect out of a population of  $(x + y + z)$ , then  $(x + y - kz)$  will not show the effect. The true value of the constant of association " $\chi^2$ " will thus be obtained from the relationship given below in which  $(x + x_1)$  is written  $\Sigma x$ , etc. This is,

$$"\chi^2" = \frac{\{[x + (1-k)z][y_1 + k_1z_1] - [x_1 + (1-k_1)z_1][y + kz]\}^2(N + N_1)}{(\Sigma x + \Sigma z - kz - k_1z_1)(\Sigma y + kz + k_1z_1)NN_1}. \quad (22)$$

The denominator of this function cannot be greatly simplified but the numerator (*num*) reduces to a relatively simple form, for:

$$\begin{aligned} \frac{num}{(N + N_1)} &= [(x + z - kz)(y_1 + k_1z_1) - (x_1 + z_1 - k_1z_1)(y + kz)]^2 \\ &= [(xy_1 + xk_1z_1 + y_1z + k_1zz_1) - (y_1kz - kk_1zz_1 - x_1y - x_1kz - yz_1 - kzz_1 + yk_1z_1 + kk_1zz_1)]^2 \\ &= [k_1z_1(x + y + z) - kz(x_1 + y_1 + z_1) + y_1(x + z) - y(x_1 + z_1)]^2 \\ &= [Nk_1z_1 - N_1kz + y_1(N - y) - y(N_1 - y_1)]^2 \\ &= [Nk_1z_1 - N_1kz + Ny_1 - N_1y]^2 \\ &= [N(k_1z_1 + y_1) - N_1(kz + y)]^2. \end{aligned} \quad (23)$$

Substituting (23) in (22),

$$"\chi^2" = \frac{[N(k_1z_1 + y_1) - N_1(kz + y)]^2(N + N_1)}{[\Sigma x + \Sigma z - (kz + k_1z_1)][\Sigma y + (kz + k_1z_1)]NN_1}. \quad (24)$$

This is, then, the general expression for determining the true value of " $\chi^2$ " when comparing the true values of  $y$  in two populations. In the absence of any information as to the values of  $k$  and  $k_1$ , it is evident that the numerical value of the true constant of association cannot be determined. It is also worthless for practical purposes, to assume the relationship (7) since this leads to a function so complex that few workers would care to derive its value. There remains, nevertheless, the fact that the true value of " $\chi^2$ " must lie within four limits, since  $k$  and  $k_1$  each have the two limits defined in (20) and (21). These four limiting values can be written down by inspection as the following, derived by substituting the given values of  $k$  and  $k_1$  in (24), and by writing in the constant  $K$ , where:

$$K = \frac{N + N_1}{NN_1}. \quad (25)$$



When  $k = k_1 = 0$ ,

$$"X^2" = K \frac{(Ny_1 - N_1y)^2}{\Sigma y(\Sigma x + \Sigma z)} \quad (26)$$

When  $k = k_1 = 1$ ,

$$\begin{aligned} "X^2" &= K \frac{[N(z_1 + y_1) - N_1(z + y)]^2}{[\Sigma x + \Sigma z - (z + z_1)][\Sigma y + (z + z_1)]} \\ &= K \frac{[N(N_1 - x_1) - N_1(N - x)]^2}{(\Sigma x + \Sigma z - z)(\Sigma y + \Sigma z)} \\ &= K \frac{(N_1x - Nx_1)^2}{\Sigma x(\Sigma y + \Sigma z)} \end{aligned} \quad (27)$$

When  $k = 1$  and  $k_1 = 0$ ,

$$\begin{aligned} "X^2" &= K \frac{[Ny_1 - N_1(y + z)]^2}{(\Sigma x + \Sigma z - z)(\Sigma y + z)} \\ &= K \frac{[N_1x - N(x_1 + z_1)]^2}{(\Sigma x + z_1)(\Sigma y + z)} \end{aligned} \quad (28)$$

When  $k = 0$  and  $k_1 = 1$ ,

$$\begin{aligned} "X^2" &= K \frac{[N(y_1 + z_1) - N_1y]^2}{(\Sigma x + \Sigma z - z_1)(\Sigma y + z_1)} \\ &= K \frac{[N_1(x + z) - Nx_1]^2}{(\Sigma x + z)(\Sigma y + z_1)} \end{aligned} \quad (29)$$

The four expressions (26), (27), (28) and (29) represent the absolute limits within which the constant of association can vary, irrespective of the absolute value of the death rate. It is very little more trouble to find the numerical value of all four than it is to find the value of the customary formula (10) since many of the terms are common. If all four values so found are greater than 4, it is evident that  $y_t$  and  $y_{t_1}$  are validly different from each other under all circumstances. Moreover, it is possible, by demonstrating which of these expressions is less than 4, to show under what conditions of death rate the comparison becomes invalid; or to prove that in no circumstances is the difference significant.

The great utility and flexibility of these formulae are best demonstrated by two examples of their application. In the first case it was desired to determine whether the injection of a certain material into the white of developing hens' eggs retarded the growth of the embryo. It was known that the injection of normal saline caused some retarda-

tion. Two series of injections were accordingly run, the first with normal saline alone, and the other with a solution of the substance under examination in normal saline. The results, in tabular form, were:

	Normal	Retarded	Dead	Total
Saline alone	41	28	5	74
Saline + reagent	30	47	7	84

Rewriting this in terms of the formulae just given, and deriving the required common terms:

$$\begin{array}{cccc}
 x = 41 & y = 28 & z = 5 & N = 74 \\
 x_1 = 30 & y_1 = 47 & z_1 = 7 & N_1 = 84 \\
 \hline
 \Sigma x = 71 & \Sigma y = 75 & \Sigma z = 12 & \Sigma N = 158
 \end{array}$$

$$K = \frac{N + N_1}{NN_1} = \frac{158}{6216} = 0.025$$

$$\begin{array}{cccc}
 N = 74 & Ny_1 = 3474 & Nx_1 = 2220 & N(x_1 + z_1) = 2738 \\
 N_1 = 84 & N_1y = 2352 & N_1x = 3444 & N_1(x + z) = 3864
 \end{array}$$

Then, if  $k = k_1 = 0$  (from 26),

$$“\chi^2” = .025 \frac{1122^2}{158} = .025 \frac{1258884}{158} = .025 \times 202 = 5.1;$$

if  $k = k_1 = 1$  (from 27),

$$“\chi^2” = .025 \frac{1224^2}{71 \times 87} = .025 \frac{1498176}{6177} = .025 \times 243 = 6.1;$$

if  $k = 1$  and  $k_1 = 0$  (from 28),

$$“\chi^2” = .025 \frac{706^2}{78 \times 80} = .025 \frac{498436}{6160} = .025 \times 80 = 2.0;$$

if  $k = 0$  and  $k_1 = 1$  (from 29),

$$“\chi^2” = .025 \frac{1644^2}{76 \times 82} = .025 \frac{2702736}{6232} = .025 \times 434 = 10.9.$$

The four limiting values of “ $\chi^2$ ” in this experiment are, therefore, 5.1, 6.1, 2.0, and 10.9; three of them are greater than the convention-

ally established limiting value of 4, which is not reached only in the case that  $k = 1$  and  $k_1 = 0$ . It is thus clear that the substance under examination gave significantly more retarded embryos than normal saline unless all the normal saline dead, and none of the reagent dead, would have been retarded had they lived. This modifying clause appears sufficiently improbable—the specific probability could be worked out—to justify regarding the difference between saline and reagent as significant.

The same experimental figures, when tested by the usual method (10), on the basis of "survivors," gives a " $\chi^2$ " value of 4.1; when tested on the basis "retarded/ not retarded" in terms of total population, the " $\chi^2$ " value is 5.2. Both results are significant and in neither case is there any justification for a conditional clause.

The second example is from a similar experiment, save that in this case the figures were:

	Normal	Retarded	Dead	Total
Saline alone	52	31	7	90
Saline + reagent	27	41	15	83

Re-writing, and deriving the common terms:

$$x = 52 \quad y = 31 \quad z = 7 \quad N = 90$$

$$x_1 = 27 \quad y_1 = 41 \quad z_1 = 15 \quad N_1 = 83$$

$$\Sigma x = 79 \quad \Sigma y = 72 \quad \Sigma z = 22 \quad \Sigma N = 173$$

$$K = \frac{N + N_1}{NN_1} = \frac{173}{7470} = 0.23$$

$$N = 90 \quad Ny_1 = 3690 \quad Nx_1 = 2340 \quad N(x_1 + z_1) = 3870$$

$$N_1 = 83 \quad N_1y = 2573 \quad N_1x = 4316 \quad N_1(x + z) = 4897$$

Then, if  $k = k_1 = 0$ ,

$$" \chi^2 " = .023 \frac{1117^2}{72 \times 101} = .023 \frac{1247689}{7272} = .023 \times 171 = 3.9;$$

if  $k = k_1 = 1$ ,

$$" \chi^2 " = .023 \frac{1886^2}{79 \times 99} = .023 \frac{23556996}{7826} = .023 \times 454 = 10.4;$$

if  $k = 1$  and  $k_1 = 0$ ,

$$“\chi^2” = .023 \frac{446^2}{94 \times 76} = .023 \frac{198916}{7426} = .023 \times 27 = 0.6;$$

if  $k = 0$  and  $k_1 = 1$ ,

$$“\chi^2” = .023 \frac{2467^2}{86 \times 97} = .023 \frac{6086089}{8342} = .023 \times 730 = 16.8.$$

This example, though the data superficially resemble that of the first, yields a very different picture. Significance is only clearly established in two cases and very doubtfully in a third; certainly it would be misleading to refer to these results as significant without qualification.

If “ $\chi^2$ ,” in this experiment, is derived from (10) in terms of survivors it is 7.9; in terms of retarded/not retarded, it is 4.0. One of these figures is clearly significant, the other is doubtful; neither gives any indication of the circumstances which attach to the acceptance of significance for either. By the methods used above, however, it is possible to state these circumstances clearly: they are that all the dead in the reagent-injected series would have been retarded had they lived. The writer feels that there are very few workers who would care, in view of this conditional clause, to claim any real significance for these results.

It is claimed, then, that the four formulae presented in this paper are better than the single formula in current biological usage. It is not claimed that the formulae presented permit the estimation of the true value of the constant of association: only that they allow the investigator to state accurately under what circumstances the constant may rise to a significant value. This is, at least, less misleading than ignoring the fact that animals may die in the course of an experiment.

## SUMMARY

In any biological population subjected to experiment, there will be three groups observed: first those showing an effect, second those not showing the effect and third, those dying.

Mathematical proof is given of the theorem that “results cannot correctly be expressed in terms of survivors unless it can be shown, either that the normal death rate is unaffected by the experimental conditions, or that the death rate is so small that it may be ignored.” A simple mathematical relationship is derived (13, page 466) which

shows, in any experiment, whether the death rate is so small that it may be ignored.

It is shown that the usual formula (10, page 464) for the derivation of the constant of association (" $\chi^2$ ") may give misleading results when used to compare two experiments in which there is an observable death rate. It is shown, however, that there are only four limiting values of " $\chi^2$ " (26, 27, 28 and 29, page 470), no matter what the death rate. These formulae do not give the true value of " $\chi^2$ ," but they permit the statement of all the possible circumstances under which it may rise to a significant value.

# Inadequacy of Diets Containing Whole-Grain Cereals Supplemented with Soybean Oil Meal for Lactation and Growth of Young Rats\*

A. H. VanLandingham and Paul B. Lyon

*From the Department of Agricultural Biochemistry,  
W. Va. Agr. Exp. Sta., Morgantown*

Received March 28, 1947

## INTRODUCTION

Recent work by Keith, Miller, Thorp and McCarty (1), Cunha, Ross, Phillips and Bohstedt (2), Krider, Fairbanks and Carroll (3), and Krider, Van Pavcke, Becker and Carroll (4) have shown that practical rations containing ground yellow corn, soybean oil meal, 5% alfalfa meal and mineral supplements are inadequate as the sole ration for brood sows under dry lot conditions. The general conclusion is that one or more as yet unidentified factors are required for normal reproduction, including lactation, in sows.

In connection with an investigation of the adequacy of certain rations containing the whole-grain cereals, corn, wheat, and oats, supplemented with solvent-extracted soybean oil meal, steam bone meal, and synthetic riboflavin for growing chickens, it was observed that, when such rations were fed weanling rats, satisfactory growth to breeding age was obtained. When the rats were continued on such rations and bred, normal appearing young were born but invariably died and, in many instances, were consumed by the mother before weaning age. It was also observed that essentially the same results were obtained when rats were grown to breeding age on a commercial rat diet<sup>1</sup> and then changed to the cereal, soybean oil meal diet at the time of mating, thus showing the effect of an inadequate diet during gestation and lactation on the performance of the young.

\* Published with the approval of the Director of the W. Va. Agr. Exp. Sta. as Scientific Paper No. 368.

<sup>1</sup> Rockland Complete Rat Diet—Arcady Farm Milling Co., Chicago, Ill.

## EXPERIMENTAL

Young weanling rats or rats grown to breeding age were placed on diet 273, composed of ground yellow corn 38, ground whole wheat 23, ground whole oats 14, alfalfa meal 2, solvent-extracted soybean oil meal 20, and steam bone meal 3, supplemented with 0.4875 g. of sodium chloride, 0.0125 g. of manganous sulphate, 0.25 g. of cod liver oil concentrate containing 4000 IU of Vitamin A and 800 A.O.A.C. units of Vitamin D/g., and 500  $\gamma$  of synthetic riboflavin/100 g. of the diet. This diet contained approximately 16% crude protein ( $N \times 6.25$ ), 3.5% ether extract, 4% crude fiber, 1.2% calcium, 0.9% phosphorus and 50 p.p.m. of manganese. The diet is adequate for satisfactory growth of normal weanling rats to maturity.

The first group of 6 female rats were placed on the diet at weaning time and were mated when 3-4 months of age. All gave birth to litters of normal weight (5.0-6.0 g./rat) with an average of 9.3 young per litter. The young rats appeared normal at birth but all died before weaning time on the 22nd day. Most of them died during the first week and many were consumed by the mother. It looked as though the young were starving from lack of milk. However, occasionally young rats were found dead with milk in their stomach.

The second group consisted of 6 female rats grown to breeding age on the Rockland Complete Rat Diet and then transferred to diet 273 at the time of mating. This group averaged 8.7 young per litter. All of the young in 4 litters died before being old enough to wean. One mother weaned her entire litter of 11 young which appeared normal but very much undersize with an average weight of only 16 g. One other mother weaned 5 young out of a litter of 7 with an average weight of 26 g. The two litters were continued on the mother's diet after weaning. The litter of 11 all died during the second month. They exhibited definite posterior paralysis and an atrophied eye condition shortly before death. Three of the litter of 5 died during the second month. Two were disposed of at the end of the second month with an average weight of 104 g.

Since the diet was relatively low in fat it was thought that choline might be needed to supplement the diet for good lactation. The diet was supplemented with 0.1% choline hydrochloride and fed to the 6 rats in the first group when mated for the second time. Four of the rats gave birth to litters with an average of 8.2 young per litter. Three of the litters died before weaning but one mother weaned 4 young with an average weight of 26 g.

Another group of 6 female rats reared on the commercial rat diet to breeding age were transferred to diet 273 supplemented with choline, gave birth to 6 litters with an average of 10.4 young per litter. Two of the litters were all dead before weaning time. Four of the mothers weaned from 2 to 6 young with an average of 4 per litter or a total of 16 rats. The young rats were unusually small, averaging only 26 g. when weaned.

The young rats weaned were not normal as indicated by the fact that 3 died during the first month after weaning. Thirteen of the young rats weighed from 26 to 60 g. when disposed of at 2 months of age.

Since diet 273 is typical of many practical rations composed of cereal grains, or a mixture of cereal grains and by-products supplemented with plant-protein concentrates and relatively small amounts of animal-protein concentrates when available,

plus bone meal and cod liver oil fed to poultry and swine, it seemed desirable to try to find a satisfactory supplement to make the ration adequate for lactation in rats. Cunha, Ross, Phillips and Bohstedt (2) reported that a ration composed of ground yellow corn 80.5, soybean meal 13.0, alfalfa meal 5.0, bone meal 0.5, limestone 0.5, and iodized salt 0.5, was inadequate for brood sows under dry lot conditions. The single addition of either crystalline riboflavin or choline as supplements to the basal ration was ineffective. Normal reproduction was obtained by supplementing the basal ration with 10% of additional alfalfa meal or 0.83 g. of pyridoxine and 0.12 lb. of soybean lecithin/100 lb. of feed. These investigators reported similar results had been obtained with rats.

Keith, Miller, Thorp and McCarty (1) reported that neither brewer's yeast,  $\beta$ -carotene, B Vitamins (nicotinic acid, thiamine, riboflavin, calcium pantothenate, and pyridoxine) corrected nutritional deficiencies of a concentrate mixture composed of corn, tankage, soybean oil meal and alfalfa meal for growing pigs. Success was obtained with 3 pigs fed 100 g. daily of vacuum-dried whole beef liver.

To study the effect of certain additions to the diet the cereal basal was modified as follows: the ground yellow corn was reduced from 38 to 35%, ground whole wheat from 23 to 20% and the ground whole oats increased from 14 to 15%, thus permitting 5% additions as supplements/100 g. of the diet. The basal diet was supplemented as follows: diet 276, 5% additional alfalfa meal; diet 277, 2% dried pig liver<sup>2</sup> and 3% dextrin; and diet 278, 5% dried brewer's yeast.<sup>3</sup>

The results obtained with the various supplements are presented in Table I. It may be observed in the case of diet 276 when 5% additional alfalfa meal was added 7 out of a total of 11 litters were weaned with an average of 4.6 young per litter. Four of the litters died before weaning time on the 22nd day after birth. When the basal diet was supplemented with 2% dried pig liver, 19 of the 21 litters were weaned with an average of 6 young per litter with an average weight of 40.4 g./rat. Two of the litters were lost before weaning time. When 5% dried brewer's yeast was added as

TABLE I

*Effect of Basal Ration Supplemented with Alfalfa Meal, Dried Pig Liver, and Dried Brewer's Yeast on the Performance of Rats During Gestation and Lactation*

Diet during gestation and lactation	Number litters born	Number young born per litter	Number litters lost	Percentage of litters lost	Number litters weaned	Average No. per litter weaned	Average weaning weight per rat
276—Basal·Alfalfa	11	7.7	4	36.4	7	4.6	27.5
277—Basal·Liver	21	10.3	2	4.8	19	6.0	40.4
278—Basal·Yeast	7	10.1	4	57.1	3	5.7	28.4

<sup>2</sup> Fresh pig liver was steamed at 100°C. for 5 minutes, ground and dried below 75°C. Juices formed were dried on ground liver.

<sup>3</sup> Fleischmann's Dry Brewer's yeast, Type 2019.



supplement, diet 278, only 3 out of a total of 7 litters were weaned with an average of 5.7 young with an average of 28.4 g./rat. Four of the litters all died before weaning time. In this experiment all litters were reduced to 8 on the third day and were weaned on the 22nd day after birth. It may also be observed in Table I that there was considerable variation in the average weight per rat weaned on the various diets. The young rats on diet 277 with the dried pig liver were decidedly larger than those on diet 276 with alfalfa meal or 278 with dried brewer's yeast as supplement.

TABLE II  
*Effect of Diet on the Growth of Young Rats after Weaning*  
(Body weight of rats at two months of age)

	Diets used		
	276 Basal-Alfalfa	277 Basal-Liver	278 Basal-Yeast
Number of male rats	10	12	7
Range in body weight	38-134 g.	170-260 g.	87-143 g.
Average body weight	86 g.	215 g.	116 g.
Number of female rats	8	21	6
Range in body weights	80-123 g.	133-166 g.	40-129 g.
Average body weights	109 g.	152 g.	68 g.

Some of the young rats weaned were continued on the same diets that the mothers received during the gestation and lactation period. In Table II are presented data showing a wide range in body weight at two months of age. The rats on diet 277 were definitely larger than on any of the other diets. The order of weights on the various diets were about the same as when the young rats were weaned.

## DISCUSSION

From the evidence presented it may be seen that rations containing the whole-grain cereals, corn, wheat, oats and solvent-extracted soybean oil meal supplemented with bone meal, crystalline riboflavin, choline hydrochloride and cod liver oil are not adequate for good lactation in rats as indicated by the large proportion of the young dying before weaning time. Also by the lack of growth in the case of young rats which lived to weaning age.

The addition of 2% dried pig's liver to the basal diet showed marked improvement in the number of young weaned as well as in the growth of the young before and after weaning. In fact we have rats fed this ration containing liver for two generations and in excellent condition. The addition of 5% alfalfa meal or 5% dried brewer's yeast showed

some improvement over the basal diet, but the diet was still inadequate for good lactation.

Preliminary studies in which parts of litters from mothers on the diet 277 supplemented with liver were exchanged for an equal number of young from mothers on diet 276 supplemented with alfalfa meal indicates that the difficulty is due to poor lactation. Either a lack of sufficient milk or a lack of some factor or factors in the milk. Further work is in progress. There seems to be no lack of ability of the young born on the basal diet to grow if transferred at an early age to mothers receiving liver in their diet.

Liver seems to occupy a strategic place in the diet for good lactation. Just what factor, or factors, in the liver is responsible for the favorable results is not known. There is no indication that either choline or riboflavin was of benefit. In fact it seems doubtful that the missing factor supplied by the liver is in the B complex.

Recently Krider, Van Poucke, Becker and Carroll (4) reported that gestation and lactation results with brood sows were significantly improved by the addition of alfalfa meal or liver extract to the basal ration containing the known B vitamins, indicating that unknown required factors were being supplied by these products. Folic acid concentrates did not improve weaning weights significantly, but pigs from sows so fed were thriftier and more vigorous than were pigs from sows fed the basal ration.

The basal diet used in this study may be criticized because of the relatively low protein level (16%). However, 2% dried pig's liver or 5% dried brewer's yeast added only about 1% additional protein. We have reason to believe that the diet including liver might be improved by the addition of a higher level of protein, and possibly fat, but it seems unlikely that protein and fat could take the place of liver.

### SUMMARY

A diet containing the whole-grain cereals, corn, wheat, oats and soybean oil meal supplemented with alfalfa meal, bone meal, choline hydrochloride and riboflavin is inadequate for good lactation and growth of young rats.

The diet is adequate for lactation if supplemented by 2% dried pig's liver, but is not adequate if supplemented by 5% additional alfalfa meal or 5% dried brewer's yeast.

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# Blood Levels in Normal Adults on a Restricted Dietary Intake of B-Complex Vitamins and Tryptophan

Charles W. Denko, Walton E. Grundy and John W. Porter

*From ASF, Medical Nutrition Laboratory, Chicago, Illinois, an installation under the jurisdiction of the Surgeon General, U. S. Army*

Received April 7, 1947

## INTRODUCTION

During a study of the excretion of B-complex vitamins by normal adults on a restricted dietary intake of B-complex vitamins and protein (1) the blood levels of some of these vitamins and tryptophan were followed. Investigators reporting on factors affecting concentration and distribution of niacin in the blood (2, 3) state that niacin levels are relatively constant, even in pellagra, while the blood pantothenic acid decreases in deficiency diseases (4). Very little information has been reported on factors influencing folic acid, biotin and tryptophan in the blood.

## EXPERIMENTAL

Seven healthy young men were kept 12 weeks on a carefully controlled "normal" diet providing liberal quantities of all nutrients, 3,200 calories, 70 g. of protein, 330 g. of carbohydrate, 174 g. of fat and sufficient minerals and vitamins to meet the recommendations of the National Research Council for moderately active men. A more detailed discussion of the subjects, their activities during the experiment, their diet and the methods of analysis has been presented in a previous paper (5). Following this control period a regimen of restricted intake was followed, during which 5 of the subjects received a diet containing 25-45% as much folic acid, biotin, pantothenic acid, niacin and tryptophan as in the control diet. The intake of protein was low, 40 g./man/day, only 3 g. of which was animal protein. Corn supplied 27% of the calories. Two control subjects received the same diet supplemented with animal protein, 45 g./day of calcium caseinate, and synthetic B-vitamins to the level in the previous control diet. The restricted diet provided daily about 3,300 calories, 40 g. protein, 380 g. of carbohydrate, 175 g. of fat and sufficient vitamin A and minerals to meet the recommendations of the National Research Council for moderately active men. The food consisted of foods frequently consumed in certain parts of the country such as corn meal in various forms (as fried mush, muffins and cookies), spaghetti, carrots, string

TABLE I  
*Daily Intake of B-Vitamins and Tryptophan*

Nutrient	Control period (12 weeks) All 7 subjects	Restricted intake (15 weeks)	
		5 Restricted subjects	2 Supplemented subjects
Thiamine, mg.	1.44	0.54	1.73
Riboflavin, mg.	1.84	0.32	1.98
Pyridoxine, mg.	1.76	1.05	4.05
Niacin, mg.	15.6	5.7	17.6
Pantothenic Acid, mg.	4.7	1.1	7.2
Biotin, $\gamma$	44	20	80
Folic Acid, $\gamma$	64	22	112
Tryptophan, mg.	832	237	682

beans, beets, gelatin, apples, pears, pineapple, salt pork, oleomargarine and sugar. For one day each week the food was analyzed to give the values in Table I.

Blood values of the vitamins and tryptophan were determined 3 times during the control period and 3 times during the 15-week period of restricted intake, once in the final week. All blood samples were obtained by venipuncture about one hour after breakfast. The blood was oxalated and stored in the refrigerator until used. These vitamins and tryptophan remained stable for at least 8 days in blood stored at 5°C. The analyses for folic acid and biotin were made microbiologically with *Lactobacillus casei* according to the method of Teply and Elvehjem (6). *L. arabinosus* was used to determine pantothenic acid by the method of Hoag and co-workers (7) modified by using crystalline vitamin solutions in place of the yeast and vitab mixtures. *L. arabinosus* was also used for niacin determination by the method of Krehl and co-workers (8) and for tryptophan by a method based on that of Woolley and Sebrell (9). Blood was given the following prior treatment before analysis: acid hydrolysis, HCl, for biotin; alkaline hydrolysis, Ba(OH)<sub>2</sub>, for tryptophan; enzymatic hydrolysis, takadiastase for pantothenic and folic acid determinations and simple water dilution for niacin. To determine distribution in the blood, whole blood and plasma levels were studied during the restricted intake period. The plasma was treated for assay the same as whole blood.

## RESULTS AND DISCUSSION

The whole blood and plasma values for folic acid, biotin, pantothenic acid, niacin and tryptophan for the control period and the period of restricted intake are given in Table II. Each average figure represents the average of 3 determinations done on all group subjects during each dietary period. The restricted group represents the 5 subjects who

TABLE II  
*Vitamin and Tryptophan Level of Whole Blood and Plasma*

	Folic acid Range-average	Biotin Range-average	Pantothenic acid Range-average	Niacin Range-average	Tryptophan Range-average
	$\gamma$ /100 ml.	$\gamma$ /100 ml.	$\gamma$ /100 ml.	mg./100 ml.	mg./100 ml.
Control Period—Normal Diet—12 Weeks Whole Blood					
Control Subjects	2.30-5.28-3.53	0.75-1.73-1.23	23-45-31	0.62-0.76-0.69	269-289-274
Restricted Subj.	1.94-2.79-2.32	0.81-1.91-1.29	16-38-29	0.56-0.82-0.65	266-320-291
Restricted Intake Period—15 Weeks Whole Blood					
Control Subjects	1.50-3.86-2.64	1.00-1.56-1.33	29-47-33	0.62-0.78-0.73	266-305-284
Restricted Subj.	1.20-1.88-1.62	0.55-1.94-1.41	18-66-31	0.53-0.72-0.66	222-322-276
Plasma					
Control Subjects	1.62-1.95-1.75	0.95-1.66-1.27	6-22-12	<0.04	106-122-114
Restricted Subj.	0.86-2.25-1.62	0.64-1.55-1.06	6-18-10	<0.04	82-103-96

received the restricted intake while the control group represents the 2 subjects who received the restricted intake plus sufficient supplement to equal or exceed consumption during the normal or control period. Tryptophan, however, was somewhat lower than in the control period. The range figures are the extreme values, minimum and maximum, found among all group subjects during each dietary period.

The blood levels in the 15th week on restricted intake were not appreciably different from those during the control period on normal diet, nor was there significant variation between the 5 subjects receiving the restricted intake and the 2 control subjects receiving a normal amount of the factors listed. Urinary excretions of these vitamins in subjects on restricted intake decreased markedly (1) while blood values remained normal and no clinical signs of deficiency were noted during the period of study. Tryptophan excretion did not fall noticeably during this study (10).

The plasma was practically devoid of niacin and contained less folic acid, biotin, pantothenic acid and tryptophan than the whole blood. Evidently the formed elements of the blood contain considerable quantities of all these factors. Apparently the blood does not reflect moderately decreased dietary intake (25-45% of normal control diet) of these factors very rapidly, at least not in the 15-week period studied.

## ACKNOWLEDGEMENT

The authors wish to express their appreciation to Lederle Laboratories, Pearl River, N. Y., for furnishing synthetic folic acid, to Merck & Co., Inc., Rahway, N. J., for furnishing synthetic biotin, to Abbot Laboratories, North Chicago, Ill., for preparing and furnishing the capsules and tablets used in supplementation; and to Miss Jane R. Spinella and Miss Marta E. Wood of this laboratory for planning the diet. It is a pleasure to acknowledge the cooperation of the following volunteer members of the Civilian Public Service on detached service for the project under the auspices of the Army Epidemiological Board: Delbert B. Blickenstaff, Edward L. Crill, Harvey E. Dibrell, Jr., Gareth W. Heisler, Roy W. Miller, John H. Smith, and Lee Smith, Jr.

## SUMMARY

1. The blood levels of folic acid, biotin, pantothenic acid, niacin and tryptophan were studied in healthy normal adults during a period on a controlled "normal" diet and during a period of restricted dietary intake (25-45% of normal levels) of these nutrients.

2. The blood values of folic acid, biotin, pantothenic acid, niacin and tryptophan did not change during this 15-week period of moderately decreased dietary intake.

3. Plasma is practically devoid of niacin and contains less folic acid, biotin, pantothenic acid and tryptophan than whole blood.

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## LETTERS TO THE EDITORS

### The Rates of Glycolysis of Glucose and Fructose in Extracts of Brain \*

Sirs:

In two preceding papers (1, 2) the rate of glycolysis in preparations of brain tissue has been investigated. The complete homogenate made with Ringer solution and "fortified" with all the necessary coenzyme factors showed a turnover rate similar to that of slices of gray matter, but declining faster, while a centrifuged extract free from structural elements gave a much higher rate which was nearly constant for about an hour.

The homogenate, moreover, exhibited the same difference of glycolytic rates between glucose, fructose and galactose as the tissue slices, but the extract did not; here fructose was glycolyzed at the same high speed as glucose while galactose was not attacked. Most of these differences could be explained by the distribution and activity of the two enzymes hexokinase and apyrase.<sup>1</sup>

The apyrase is strongly adsorbed on the structural elements, the complete homogenate containing at least 10 times as much enzyme before centrifugation as afterwards. The total amount of ATP contained in fresh brain tissue (3) would, in the absence of sugars, be split in 10 seconds at 38° C. by the apyrase liberated from the same tissue by homogenization. This does not happen in the extract.

If the ATP is continuously replaced in the diluted homogenate, either by adding it afresh every two minutes (1), or by supplementing the system with phosphocreatine or hexosediphosphate as P-donors (2), turnover rates are obtained approaching that in the extract and the difference between glucose and fructose nearly disappears. From this we concluded that the difference is due to the low concentration

\* This work was supported by grants of the Baird Foundation and Dazian Foundation. It was carried out with the technical assistance of Mrs. Jean R. Wilson.

<sup>1</sup> Abbreviations: ATP = adenosinetriphosphate; ADP = adenosinediphosphate; Apyrase = adenylpyrophosphatase, splitting both labile P groups; HDP = hexosediphosphate.



of ATP which occurs in the homogenate. At high ATP concentrations the affinity of the adsorbed hexokinase for fructose is the same as for glucose but is much less at low concentrations of ATP.

We encountered difficulties, however, in proving this point for the dissolved hexokinase in the extract; even with low ATP concentrations the rates of turnover of glucose and fructose were, in general, about the same. We have now found that this depended on the relatively large amount of HDP used for priming the reaction. Without any added HDP the glycolysis does not start at all with the low concentration of ATP. But if the added amount of HDP is appreciably larger than the added amount of ATP, some of the adenylic compounds still existing in the extract are rephosphorylated and the critical concentration of ATP is overstepped. Then the difference between the two hexoses disappears. With our dilutions of the extract,  $1 \times 10^{-4}$  to  $2 \times 10^{-4}$  M HDP (6–12 $\gamma$  P/cc.) and  $0.3 \times 10^{-4}$  to  $1.5 \times 10^{-4}$  M ATP (2–9 $\gamma$  pyro-P/cc.) are suited to demonstrate the difference between glucose and fructose in the extract (Fig. 1). This relationship remains the same with pro-

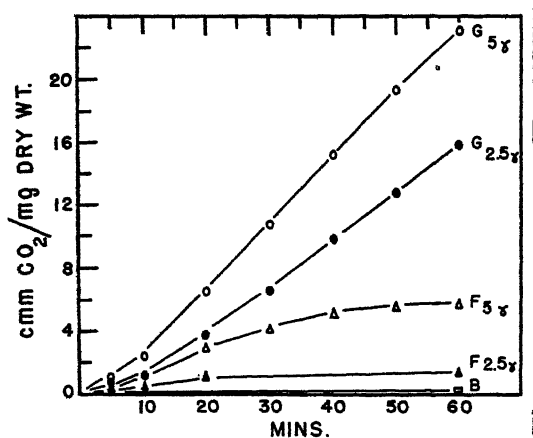


FIG. 1.—Glycolysis of glucose and fructose with very low ATP concentrations. All samples contain 0.95 cc. total vol., 0.3 cc. extract (1 part tissue to 3.3 parts modified Ringer solution),  $1 \times 10^{-2}$  M glutathione,  $0.3 \times 10^{-2}$  M NaHCO<sub>3</sub>,  $5 \times 10^{-3}$  M phosphate, 0.3 mg. DPN, HDP with 7 $\gamma$  P and 2 mg. sugar.

B □ Blank

G<sub>5γ</sub> ○—○ 1 mg. glucose 5 $\gamma$  pyro-P of ATP.

F<sub>5γ</sub> △—△ 1 mg. fructose 5 $\gamma$  pyro-P of ATP.

G<sub>2.5γ</sub> ●—● 1 mg. glucose 2.5 $\gamma$  pyro-P of ATP.

F<sub>2.5γ</sub> ▲—▲ 1 mg. fructose 2.5 $\gamma$  pyro-P of ATP.

longed centrifugation of the extract although the total activity declines. For other dilutions of the extract the critical concentration of ATP and HDP is somewhat different and must be tried out for every set up, since the phenomenon obtains only in a narrow range.

The finer mechanism of the reaction between ATP and sugars must be left open, especially as to whether there is only one hexokinase responsible for the reaction of different sugars and as to whether ATP and ADP have different affinities, *etc.* Moreover, the influence of hormones as exemplified by the action of pituitary extract and insulin (4) may change the affinity of the various sugars for ATP.

However, our present observations explain in the main the glycolytic rates as found in the intact cell. Quite obviously the acting concentration of ATP is here only a few *per cent* of the total concentration found by analysis. The basic principle which we had found previously only in the homogenate is now generally established, *viz.*, with decreasing concentrations of ATP the affinity of the hexokinase of brain for fructose is lowered much more than that for glucose.

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Department of Physiological Chemistry,  
School of Medicine,  
University of Pennsylvania,  
Philadelphia, Pa.

OTTO MEYERHOF

March 25, 1947.

#### A New Antihistaminic: N,N-dimethyl-N'(2-pyridyl)-N'-(2-thienylmethyl)-ethylenediamine Hydrochloride.

Since the French authors Fourneau, Bovet, Staub, Halpern and Mosnier prepared active antihistaminic compounds—as reviewed by Bovet (1)—the work of numerous investigators has been centered around the study of new drugs with this specific action. In particular, two compounds— $\beta$ -dimethylaminoethylbenzhydryl ether (Benadryl), described by Loew, Kaiser and Moore (2), and N,N-dimethyl-N'-(2-pyridyl)-N'-(benzyl)-ethylenediamine (Pyribenzamine), described

by Mayer, Huttner and Scholz (3)—have been studied extensively, both from the pharmacological and clinical standpoint.

Among a series of new compounds, W53 = N,N-dimethyl-N'-(2-pyridyl)-N'-(2-thienylmethyl)-ethylenediamine hydrochloride, possessing a high antihistaminic activity, has been synthesized in these laboratories (4).

The minimal subcutaneous active dose of W53, which protects guinea pigs against 2–5 lethal doses of histamine injected intravenously, is 0.05–0.10 mg./kg. Protection against high doses of intravenous histamine (150–200 LD) is obtained by increasing the dosage of W53 to 10–15 mg./kg.

Protective activity against the bronchial asthma of guinea pigs, induced by exposure to nebulized histamine phosphate, is obtained with doses of 0.025 mg./kg. of W53 given subcutaneously.

Experiments on the isolated guinea-pig intestine showed that W53 in dilution of 1:2,000,000 inhibits the contractions produced by 1  $\gamma$  histamine.

The symptoms following the administration of W53 to mice, rabbits and guinea pigs are similar to those recorded for Antergan, Neo-antergan, Benadryl and Pyribenzamine; the pharmacological experiments (circulation) on anesthetized dogs also evidenced corresponding similarities between these drugs. From a quantitative toxicological standpoint, the convulsive and lethal doses obtained with W 53 are in the order found for the antihistaminics mentioned.

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*The Warner Institute for Therapeutic Research,  
New York, N. Y.*

*April 24, 1947.*

N. ERCOLI  
R. J. SCHACHTER  
F. LEONARD  
U. V. SOLMSEN

## Book Reviews

**Carbohydrate Metabolism. Correlation of Physiological, Biochemical, and Clinical Aspects.** By SAMUEL SOSKIN, M.D., AND RACHMIEL LEVINE, M.D. The University of Chicago Press, Chicago, Illinois. viii + 315 pp. Price \$6.00.

This book is essentially a treatise on diabetes and the biochemical background of this disease, written by authors who are well known for their experimental research work in this field.

It is not the fault of the writers, but a curious failure of cooperation between biochemistry and experimental pathology that, in spite of our progress in the field of carbohydrate metabolism and our nearly complete knowledge of the oxidative and anaerobic intermediary steps, we do not know the exact locus of attack of insulin 50 years after the discovery of pancreatic diabetes by Mehring and Minkowski and 25 years after the isolation of insulin. Even the question as to whether the primary reason for diabetic hyperglycemia is over-production of glucose or lack of utilization, debated since the time of Minkowski, is not definitely answered today.

This reviewer cannot quite accept the argument of the authors who stress the role of over-production of glucose from non-carbohydrate sources as the main disturbance. On the other hand, their conjecture that the phosphorylation of sugar is impaired seems now fully justified by the discovery from Cori's laboratory, not yet incorporated in this book, that the reaction between glucose and adenosinetriphosphate (ATP) in the presence of hexokinase is slowed down by the anterior pituitary hormone and this inhibition relieved by insulin. On account of the integration of all the functions of the body by nervous and hormonal control, lack of insulin should first impair the utilization of carbohydrate and the ensuing hyperglycemia and glycosuria with loss of sugar from the body would stimulate gluconeogenesis. It must also be emphasized that impaired function should not be identified with loss of function. The complete inability of a cell to metabolize sugar would be surely incompatible with life. Decreased reactivity, however, is very well conceivable as the clue to the pathogenesis of diabetes.

The main part of the book, devoted to the study of diabetes, from p. 75 to the end (p. 300), is well worth reading. The role of the many hormones in intermediary metabolism is exhaustively expounded with ample references. On the other hand, the biochemistry and energetics of carbohydrate metabolism is treated rather stingily in less than 50 pages. This includes "the enzymatic machinery of carbohydrate metabolism," the "intermediary steps," anaerobic and aerobic, "the liberation and transfer of energy," and "the use of energy for muscular contraction." Thanks to this shortness the presentation of our knowledge in this fundamental realm is often contracted to mere schemes and the literature quoted in this section consists mainly of English and American reviews instead of the original papers. This sometimes leads to the impression that the achievements are credited to the reviewers instead of to the real

discoverers. So far as this writer is aware, the schemes are generally correct with only minor lapses (e.g., the formula of 3-phosphoglyceraldehyde, p. 51, is incorrectly written, and on p. 192, where the scheme is repeated, 1,3-diphosphoglyceric acid is left out). A more serious error occurs in the section on energy transfer. The authors do not clearly distinguish between change of free energy ( $\Delta F$ ) and of total energy ( $\Delta H$ ). They explain the terminology of "exergonic" and "endergonic" reactions as a more precise expression than "exothermic" and "endothermic" because of the constancy of temperature of the homoiothermic animal. Also the difference between a heat engine (combustion motor or steam engine) and a chemodynamic machine (like storage battery plus electric motor) is not explained in unambiguous terms. This part should be revised carefully before a new printing.

All in all, the physician and pathologist will derive useful knowledge, and the biochemical reader also will appreciate the large amount of experimental work discussed in the main chapters of the book.

OTTO MEYERHOF, Philadelphia, Pa.

**The Chemical Kinetics of the Bacterial Cell.** By C. N. HINSHELWOOD, Dr. Lee's Professor of Chemistry, at the University of Oxford, Oxford. Clarendon Press, 1946. viii + 283 pp. Price \$6.75.

The author of the well-known book "The Kinetics of Chemical Change" (1940) is one of those modern physicochemists who have turned their interest to biology and have tried to find out whether the application of physical laws to biology will be more successful in their hands than in the hands of professional biologists. The author, having acquired a large personal experience for many years from experiments on bacterial growth, tries, in this new book, a general review of the problem of the growth of bacteria, adaptation to drugs and to new sources of carbon or nitrogen, mutation, selection, and many other subjects, and to generalize the ideas to living matter in general. The author's own remark in the preface: "the book has been written (just) from a desire to place certain matters in relation to one another . . . and to see what the composition looked like," to be sure, is an all too modest understatement. Rather is the book a very systematic survey of the problem of growth. Mathematical formulation plays a great, perhaps too great and too formal, a role. An important thesis about the problem of self-duplication of living matter is the formula: enzyme + substrate = more enzyme + product, in analogy to the inorganic system: catalyst (e.g., CaO) + substrate ( $\text{CaCO}_3$ ) = more catalyst (CaO) +  $\text{CO}_2$ . Although the result as to the final problem: What is life? is rather restricted, one must be grateful to a scientist of this rank for having the courage to build up a valuable frame for future work. This book will have its definite place in the history of man's exploration into the nature of life, in addition to the interesting survey of the experiments with bacteria.

L. MICHAELIS, New York, N. Y.

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VOLUME 14



1947

ACADEMIC PRESS INC. PUBLISHERS  
NEW YORK, N. Y.

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# A Galactogen from Beef Lung<sup>1</sup>

M. L. Wolfrom, D. I. Weisblat<sup>1</sup> and J. V. Karabinos<sup>2</sup>

*Chemical Laboratory of The Ohio State University, Columbus, Ohio*

and

O. Keller

*Scientific Department, Hoffmann-La Roche, Inc., Nutley, New Jersey*

Received February 10, 1947

## INTRODUCTION

Numerous workers have isolated from natural sources, various amorphous carbohydrate fractions which gave a high yield of D-galactose on hydrolysis. Many such products have been characterized initially as a D-galactose polysaccharide or galactan, only to have such a description revised later when a more complete investigation disclosed the presence of hydrolytic products other than D-galactose. Recently, Hirst and co-workers (1, 2) have tended somewhat to reverse this trend and they consider that their methylation studies indicate the presence of a galactan in admixture with other polysaccharides, such as pectin and araban. Details concerning the nature of such a galactan have not yet been published.

Hassid (3) has isolated from a marine red alga, *Iridaea laminariodes*, a galactan acid sulfate ( $R-O-SO_3-OH$ ) in the form of its sodium salt and has presented evidence that the sugar moiety is D-galactose alone. A closely related galactan has been reported from the alga *Dilsea edulis* (4). A well-characterized galactan of apparently low molecular weight is that produced by the mold *Penicillium charlesii* G. Smith and designated galacto-carolose (5, 6).

In the animal world, it has been found that the mucoitinsulfuric acid extractable from the snail *Helix pomatia* is accompanied by varying amounts of glycogen and a water-soluble galactan ( $[\alpha]_D$  ca.  $-20^\circ$ , water), designated by May (7) as "galactogen" by analogy with "glycogen." This polysaccharide was perhaps first isolated by Hammarsten (8) and was further investigated by other workers (7, 9-13). It was first

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<sup>1</sup> Dedicated to Professor Carl Neuberg on his 70th birthday.

<sup>2</sup> Hoffmann-La Roche Research Associate (D. I. W.) and Fellow (J. V. K.) of The Ohio State University Research Foundation (Project 83).

adequately purified and characterized by May (7, 10, 11) who believed it to be present in several other cold-blooded animals. Bell and Baldwin (14) proved the presence in this substance of some L-galactose by the isolation of DL-galactobenzimidazole according to the procedure of Moore and Link (15).

In the experimental work herein described, we have isolated from beef lung a galactogen ( $[\alpha]_D^{25} + 18^\circ$ , water) which is different from that obtained from the snail.

## EXPERIMENTAL

### *Isolation and Characterization*

A crude heparin (neutral sodium salt) preparation from beef lung was precipitated as the insoluble benzidine salt which was removed by filtration. From the mother liquor a precipitate was obtained on the addition of an excess of ethanol. This precipitate (5 g.) was dissolved in 66 ml. of warm water, treated with 34 ml. of glacial acetic acid, and filtered through Celite (Johns-Manville Co., New York, N. Y.). To the clear filtrate was then added a hot solution of 5 g. of freshly distilled benzidine in 26 ml. of 50% acetic acid and the whole allowed to cool to room temperature, filtered through Celite and the filtrate treated with 5 volumes of ethanol (95%). The precipitate was separated by centrifugation and washed with ethanol. It was redissolved in 200 ml. of hot water, cooled to  $0^\circ\text{C}$ . and filtered through Celite. The clear filtrate was added slowly to 5 volumes of ethanol with vigorous stirring and the gelatinous precipitate collected by centrifugation and washed with ethanol and ether; yield 4.1 g.

A portion of the above product (200 mg.) was dissolved in 10 ml. of water and passed through an acid cation exchange column (Amberlite IR-100; Resinous Products and Chemical Co., Philadelphia, Pa.) followed by passage through an anion acceptor column (Amberlite IR-4). The effluent and washings were concentrated to dryness at room temperature and the residue dissolved in 10 ml. of water and precipitated with 4 volumes of ethanol. The centrifugally separated and washed (with ethanol and ether) product was a colorless, amorphous, tasteless powder;  $[\alpha]_D^{25} + 18^\circ$  (c 2, water). On heating in a capillary tube it darkened slightly at  $162^\circ$ , became brown at  $185^\circ$ , and decomposed at  $227\text{--}232^\circ\text{C}$ . For analysis the substance was dried at  $100^\circ\text{C}$ . over phosphorus pentoxide under reduced pressure.

*Anal.* Calcd. for  $\text{C}_6\text{H}_{10}\text{O}_5$ : C, 44.44; H, 6.22. Found: C, 44.19; H, 6.29; ash, 0.4; S, absent; P, present. Calcd. to an ash-free basis: C, 44.36; H, 6.32.

The material was soluble in water, giving a clear solution, and was practically insoluble in the common organic solvents. An aqueous solution (25.0 g./1000 g. of water) depressed the freezing point of water very slightly ( $< 0.03^\circ\text{C}$ .); relative viscosity 1.43 ( $25^\circ$ , c 5, water). The substance was very slightly reducing, showing a reduction of 0.55% (calcd. as galactose) by the Hagedorn and Jensen method. It possessed no measurable blood anticoagulant activity.

The product did not change the color of an iodine solution. It yielded a positive Molisch test for carbohydrates and exhibited a positive reaction with May's (7) alkaline copper reagent (a galactogen test). Negative qualitative tests were obtained for ketoses (16), pentoses (17), 6-desoxyhexoses (18), uronic acids (19) and mannose (20). The substance produced mucic acid on  $\text{HNO}_3$  oxidation by the procedure of van der Haar (21); m. p.  $210^\circ\text{C}$ . (dec.) unchanged on admixture with an authentic specimen of mucic acid. The filtrate from the mucic acid test, on concentration to dryness, neutralization with potassium carbonate and acidification with an excess of acetic acid, gave no trace of potassium acid saccharate; the probable absence of glucose was thus indicated (22, 23). The absence of glycogen was demonstrated by recovering the material (by ethanol precipitation), unchanged in specific rotation ( $+18^\circ$  in water,  $c$  2,  $25^\circ\text{C}$ .), after digestion in aqueous solution with filtered saliva.

### *Hydrolysis*

The reducing value by the Hagedorn and Jensen method reached a constant value of *ca.* 110% (calcd. as galactose) in twelve hours on hydrolysis ( $c$ , 0.33 g. substance/100 ml. of solution) with 0.5  $N$   $\text{H}_2\text{SO}_4$  at  $98^\circ\text{C}$ .

An amount of 1.00 g. of the polysaccharide was dissolved in 33.3 ml. of 0.5  $N$   $\text{HCl}$  and the solution heated over a boiling water bath. The heating was interrupted at various intervals, the solution cooled rapidly to room temperature ( $22^\circ\text{C}$ .) and the rotation ( $D$  line) observed. The specific rotation (initial  $+18^\circ$ ) was observed to increase rapidly in the dextro direction until the value  $+62^\circ$  was attained after 6 hours. The heating was continued for an additional 6 hours but the specific rotation remained constant. No appreciable darkening of the solution took place. The  $\text{HCl}$  was removed with an excess of  $\text{Ag}_2\text{CO}_3$  and the filtered solution treated with  $\text{H}_2\text{S}$  and then with *ca.* 200 mg. of activated charcoal, and again filtered. The clear, colorless solution was concentrated under reduced pressure ( $40$ – $45^\circ\text{C}$ .) to a sirup; yield 1.1 g.,  $[\alpha]_D^{25} + 68.5^\circ$  (water). The sirup crystallized slowly and, after trituration with cold ethanol, was filtered and washed with ethanol; yield 0.60 g. (I),  $[\alpha]_D^{25} + 80^\circ$  ( $c$  2, water). The generally accepted value for the equilibrium specific rotation of  $D$ -galactose is  $+80.5^\circ$  ( $20^\circ\text{C}$ .,  $c$  5, water,  $D$  line). The filtrate and washings were combined and precipitated with ether while cooling; yield 0.23 g. of partially crystalline material,  $[\alpha]_D^{25} + 72^\circ$  ( $c$  2, water), mucic acid test positive. The combined filtrate and washings were concentrated to dryness to yield a slightly colored sirup which was rubbed to an amorphous powder with an alcohol-ether (1:3) mixture; yield 0.12 g.,  $[\alpha]_D^{25} + 67^\circ$  ( $c$  2, water), mucic acid test positive.

A portion of lot I above was mercaptalated according to the general procedure of Fischer (24). The material (150 mg.) was dissolved in 0.5 ml. of cold, concentrated HCl ( $d$  1.19), treated with ethyl mercaptan (0.5 ml.) and the whole shaken in an ice bath until crystallization occurred. Ice water (1 ml.) was then added and cooling maintained to complete the crystallization. The crystals were removed by filtration and washed with ice water followed by alcohol-ether (1:3); yield 150 mg. Recrystallization was effected from the minimum of hot ethanol; yield 120 mg., m. p. 140–141°C., unchanged on admixture with an authentic specimen of D-galactosedithylmercaptal of like melting point.

#### *Chromatography of the Galactogen Hydrolyzate*

D-Galactose, L-galactose and the galactogen hydrolyzate (several mg. of each) were chromatographed separately on Silene EF-Celite and on clay (Florex XXX) according to the procedures previously published (25, 26). In all cases only one significant zone was detected on the chromatograms and this zone was in the position in the adsorption series previously established for D-galactose. A reducing sugar procedure (Hagedorn-Jensen) indicated that this zone contained 95% (104% of the galactogen hydrolyzed) of the reducing value of the hydrolyzate in the case of the Silene EF adsorbent and 91% (100% of the galactogen hydrolyzed) in the case of the clay adsorbent. A very small zone at the top of the column was detectable with the former adsorbent and this zone contained 3% of the reducing value of the hydrolyzate. The material constituting this top zone undoubtedly represented polymeric impurities formed during the acid hydrolysis.

#### DISCUSSION

A polysaccharide accompanies the heparin fraction from beef lung and is separable from it. It is an amorphous, tasteless powder that dissolves readily in water to give a clear solution which does not alter the color of an iodine solution. Like the snail galactogen it gives a precipitate on boiling with May's (7) alkaline copper reagent. It was recovered unchanged in rotation after digestion with salivary enzymes, thus demonstrating the absence of glycogen. Admixed ash was separated by ion exchange columns and the resultant small ash (0.4%) still present contained phosphate. Thus, very probably, a small amount

of phosphate ester is an integral part of the molecule. The elementary analysis establishes a  $C_6H_{10}O_5$  formulation, and a negligible freezing point depression, significant solution viscosity and low reduction support a polysaccharide structure. The rotation of the substance,  $[\alpha]_D^{25} + 18^\circ$  (c 2, water), sharply differentiates it from May's galactogen which has a specific rotation under like conditions of  $-20^\circ$ . It also distinguishes it from the more dextrorotatory galactans from marine algae (3, 4) and the strongly levorotatory galacto-carolose (5, 6).

Acid hydrolysis gave the calculated amount of a hexose as measured by reducing value. This hexose was demonstrated to be D-galactose by isolation in crystalline condition in high yield and by the preparation of two crystalline derivatives, mucic acid and the diethyl mercaptal. No other sugar was found to be present, although a search was made by qualitative, chromatographic and isolation methods. The absence of L-galactose is not demonstrated and the slightly low dextrorotation of the hydrolyzate indicates but does not prove its possible presence. It is the experience of the authors that none of the published methods for the quantitative assay of galactose attain their objective. In the present work, the hydrolyzate was chromatographed on both calcium acid silicate (25) and clay (26). Only one significant zone was found in each case and this was in the position on the chromatogram previously established for galactose. An amount of 91–95% of the hydrolyzate (100–104% of the galactogen hydrolyzed) was present in this zone.

#### ACKNOWLEDGMENT

We are pleased to acknowledge the counsel of Dr. H. M. Wuest and Dr. J. Lee of Hoffmann-La Roche, Inc. The assistance of Mr. F. A. H. Rice of the Columbus Laboratory in some of the experimental work is gratefully acknowledged.

#### SUMMARY

A dextrorotatory galactogen has been isolated from beef lung.

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# Action of Human Serum on the Growing Plant <sup>1</sup>

Henry K. Wachtel

*From the Cancer Research Laboratories, Fordham University, New York 58, New York*

Received January 24, 1947

## INTRODUCTION

The possibility of obtaining substances from animal organs which influence the growth of plants was studied by Berrier (1, 2). He produced auxin-like substances from organs of *Discoglossus pictus* and from guinea pigs.

In our experiments we extracted animal and human organs successively with acetone, ethyl ether, physiological salt solution, and a 0.05% solution of sodium carbonate, and tested the growth activity of the individual extracts on germinated cardamine (cress) seedlings.

Of all the important organs extracted, only the white substance of the brain and yellow bone marrow of old cattle did not influence seedling growth (3, 4). All other organs contain water-soluble and lipid growth-active factors. In the extracts, the growth-inhibiting activity is usually prevalent, excepting in those from anterior pituitary which have strong growth-promoting properties.

In further experiments we found that some, but not all, of the extracts which influenced plant growth affected the growth of young rats similarly (5). Hence lipid growth factors extracted from both lobes of the pituitary gland and found to be active with young rats, may be more expediently studied on the plant.

## EXPERIMENTAL

Continuing our studies we investigated human sera as to their influence on growth. In our experiments we used cardamine (cress) seeds germinated and grown on wet filter paper. The growth of the seedlings was observed after serum or its extract was added to the nutrient

<sup>1</sup> Dedicated to Professor Carl Neuberg on his 70th birthday.



medium (tap water). The details of the method were reported previously (6, 7). The seeds were obtained from P. Henderson and Co., New York (Extra curled cress No. 380).

Fifty random fresh human sera (normal and pathological) were examined after dilution with tap water.

The addition of serum to the nutrient medium in which the seedlings are growing, produces marked promotion of growth of the plant as compared with the growth of controls growing in tap water only. The growth-promoting effect is usually recognizable even at a serum dilution of 1:200. The lowest concentration which may produce growth promotion varies with the particular serum. Of the sera examined by us, in 8% the lowest limit was a dilution of 1:200; in 32% the dilution was 1:300; in 32%, 1:400 and in 28%, 1:600 as the limit for the growth-promoting effect.

Boiling of the serum-water mixture examined brought about a partial loss of the growth-promoting activity of the mixture. For instance, serum previously promoting growth at a dilution of 1:400, after boiling promoted growth only at 1:200.

In further experiments the serum-water mixture was extracted with ethyl ether. No marked changes in the growth-promoting activity were noted in the ether-extracted mixture. The ether extract did not influence the growth in 68% of the sera examined. In 32% of the sera the addition of the evaporated ether extract to the nutrient medium was followed by retardation of seedling growth. The retardation became manifest in concentrations of the extract corresponding to serum dilutions 5-20 times higher than the lowest limit of growth-promotion observed in the unextracted serum.

The boiled serum-water mixture was also extracted with ethyl ether. No marked changes in the growth-promoting activity were noted in the extracted mixture. The ether extract of the boiled mixture did not influence the growth in about 50% of the sera examined. This group included all sera which in the unboiled state yielded growth-retarding ether extracts. In 50% of the sera the addition of the evaporated ether extract to the nutrient medium was followed by marked promotion of seedling growth. The observed growth-promotion was manifest in concentrations of the ether extract corresponding with serum dilutions representing the lowest limit of growth promotion in the non-extracted boiled serum.

The above experiments indicate that human serum contains a

number of growth-influencing factors. A water-soluble growth-promoting factor is present, the activity of which is partially destroyed by boiling; an ether-soluble growth-inhibiting factor is present which is destroyed by boiling; and a growth-promoting heat-stable ether-soluble factor may be detected after the ether-soluble growth-inhibiting factor has been destroyed by heat.

The remarkable circumstance that the particular sera differ greatly in their content of growth-influencing factors suggests that these differences correspond with certain aspects of body metabolism. Further investigation is in progress to elucidate the relation between the state of the body and the differences existing in the content of growth factors in the sera.

#### SUMMARY

Human serum contains a number of growth-influencing factors active on the germinated plant. A water-soluble growth-promoting factor is present, the activity of which is partially destroyed by boiling; an ether-soluble growth-inhibiting factor is present which is destroyed by boiling; and a growth-promoting heat-stable ether-soluble factor may be detected after the ether-soluble growth-inhibiting factor has been destroyed by heat. The strength of the growth-influences differs markedly in the various sera.

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# The Conversion of 2,3-Butylene Glycol to Acetylmethylcarbinol in Bacterial Fermentations<sup>1,2</sup>

David Paretsky and C. H. Werkman

*From the Bacteriology Section, Iowa Agricultural  
Experiment Station, Ames, Iowa*

Received February 17, 1947

## INTRODUCTION

Many bacteria oxidize 2,3-butyleneglycol to acetylmethylcarbinol. Walpole (1) found that AMC was formed in concentrations 5-6 times greater when *Aerobacter aerogenes* was grown in a medium of 2,3-butyleneglycol and peptone water through which a current of oxygen was passed than in the unoxygenated medium. Lemoigne (2) found that *Bacillus proteus* in a glucose-peptone medium first gave rise to an increase in glycol followed by a decrease with a simultaneous increase of AMC. Werkman (3) found AMC formed by species of *Aerobacter* from a medium of 2,3-butyleneglycol, phosphate, and ammonium sulfate. Stahly and Werkman (4) found that 2,3-BG formed by *Aerobacillus polymyxa* was oxidized to AMC when the  $E_H$  was sufficiently high. Verhave (5), in a patented process, found *Aerobacter* formed AMC from 2,3-BG under aeration when inoculated in a medium containing either saccharified mash, beet sugar or molasses. Kluyver and Scheffer (6) also have a patent on a similar process. Paretsky, Wood and Werkman (7) added "resting" *A. indologenes* cells to solutions of 2,3-BG and found that 40-60% of the glycol was oxidized to AMC.

The present paper is concerned with the studies on the production of acetylmethylcarbinol as a major product in the fermentation of glucose by *A. aerogenes*.

## METHODS

The organism used was *Aerobacter aerogenes* 199, obtained from the Northern Regional Research Laboratory.

The medium consisted of 10% corn sugar, 0.4%  $(\text{NH}_4)_2\text{SO}_4$ , 10% tap water, 0.5%  $\text{K}_2\text{HPO}_4$ , 1.0%  $\text{CaCO}_3$  and distilled water. It was autoclaved at 15 pounds for 30 minutes. The phosphate was dissolved in distilled water, adjusted to pH 6.8, auto-

<sup>1</sup> Dedicated to Professor Carl Neuberg on his 70th birthday.

<sup>2</sup> Journal Paper No. J-1431 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project No. 975.

claved at 15 pounds for 20 minutes and aseptically added to the basal medium. Dry  $\text{CaCO}_3$  was also autoclaved separately and aseptically added. The corn sugar contained approximately 92% glucose.

The stock solution of methylene blue contained 1 g. of the air-dried ( $110^\circ\text{C}$ .) hydrochloride in 1000 ml. of distilled water. The autoclaved methylene blue solution was added aseptically.

*Inoculation.* A 10% aliquot of the medium was transferred aseptically to a sterile flask. *A. aerogenes* was inoculated into this medium and incubated at  $30^\circ\text{C}$ . for 18 hours. This aliquot was then added back to the sterile medium.

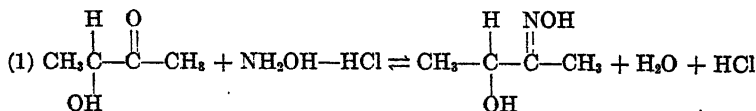
Aeration of the medium was accomplished by passing air through tubes of sterile cotton and  $\frac{3}{4}$  inch aerating stones. The procedure and tanks used for the pressure-aeration experiments were those used by Brewer *et al.* (8). Unless otherwise indicated, fermentations were conducted in 6-liter Pyrex florence flasks.

Glucose was determined according to Munson and Walker (9). The cells were precipitated by 20% phosphotungstic acid. Since AMC reduces Fehling's solution, a correction was made (10).

Acetylmethylcarbinol in the fermentation liquor was determined by oxidation with  $\text{FeCl}_3$  (11).

2,3-Butylene glycol was determined by a modification of the method of Brockmann and Werkman (12). Ten g. of anhydrous  $\text{Na}_2\text{SO}_4$  were added to a 50 ml. aliquot of the fermentation liquor. A little dry phenolphthalein was added, and 10 *N* NaOH was added until the liquor became alkaline. The liquor was distilled until the residue was saturated with  $\text{Na}_2\text{SO}_4$ . Steam distillation was then begun and 12–14 volumes distilled into a 300 ml. volumetric flask. An aliquot of the distillate (containing not more than 0.6 mM of glycol) was oxidized by  $\text{KIO}_4\text{-H}_2\text{SO}_4$  reagent, and the resulting aldehyde distilled into freshly prepared 1.5%  $\text{NaHSO}_3$  solution. Excess bisulfite was removed by iodine; the aldehyde-bisulfite complex was broken by the addition of dry  $\text{NaHCO}_3$ . Liberated bisulfite was determined by titration with dilute iodine solution. Since each molecule of AMC yields one molecule of aldehyde upon periodic acid oxidation, the glycol value must be corrected by subtracting  $\frac{1}{2}$  the value of carbinol from the observed value of carbinol plus glycol.

The AMC carried over during the steam distillation of the fermentation liquor was determined as follows: 10–20 ml. of approximately 0.2 *M*  $\text{NH}_4\text{OH}\cdot\text{HCl}$  were added to a 50 ml. Erlenmeyer flask and one drop of a 1% solution of bromphenol blue was added as an indicator. The  $\text{NH}_4\text{OH}\cdot\text{HCl}$  solution was partly neutralized by adding 0.05 *N* alkali to the first lavender color. Two drops of the bromphenol blue indicator were added to a 50.0 ml. aliquot of the steam distillate, and this portion was partially neutralized to the first light lavender end point. The  $\text{NH}_4\text{OH}\cdot\text{HCl}$  solution was then added to the aliquot of the steam distillate and, after 30–45 minutes, was titrated with 0.05 *N* NaOH to the same end point.



or, mM AMC = ml. NaOH  $\times$  normality NaOH.

This method is simple, sensitive, and has been found to be as accurate as the  $\text{FeCl}_3$  oxidation method. Its use is limited to colorless, dilute solutions; compounds containing a keto group interfere. The method is based on the determination of 2,3-butylene glycol by Brockmann and Werkmann (12).

## EXPERIMENTAL

### *Effect of Methylene Blue*

The effect of methylene blue on the fermentation of glucose by *A. aerogenes* was investigated, using a procedure tending to produce a higher redox potential in the medium by vigorous aeration. At intervals of 48, 72, and 84 hours after inoculation, sterile 70 ml. portions of the 1/1000 methylene blue stock solution were aseptically added to the flasks. After the fermentations had proceeded 96 hours, samples were withdrawn for pH determinations and concentrated  $\text{H}_2\text{SO}_4$  immediately added to the residual liquor. The liquors were then analyzed. Typical results are given in Table I.

TABLE I  
*Effect of Methylene Blue on Acetylmethylcarbinol Production*

Time of addition of methylene blue after inoculation	Acetylmethylcarbinol	2,3-Butylene glycol	AMC+2,3-BG	AMC:2,3-BG
<i>Hrs.</i>	<i>mM</i>	<i>mM</i>	<i>mM</i>	
0	17.8	55.2	73.0	0.32
48	22.9	53.0	75.9	0.43
72	27.8	50.6	78.4	0.55
84	30.4	44.3	74.7	0.69

Products in  $\text{mM}/100 \text{ mM}$  glucose fermented.

Time of fermentation: 96 hours.

Methylene blue, final concentration: 1/30,000.

Final pH values of the fermentations were between 5.6 and 5.9 as determined potentiometrically. The addition of methylene blue results in a higher AMC: 2,3-BG ratio, and better conversion to the carbinol is obtained when the methylene blue is added toward the end of the fermentation.

### *Effect of Pressure Aeration*

Brewer *et al.* (8) have shown that pressure aeration in butter cultures and in the fermentations of citric acid by streptococci will increase the

yields of diacetyl several hundred *per cent.* They found slight increases in AMC values. Mickelson and Werkman studied the effect of pressure aeration on the dissimilation of glucose by *A. indologenes* and found that AMC increases at the expense of the butylene glycol (13).

The effect of aeration under pressure on the fermentation of glucose by *A. aerogenes* was determined. Tank oxygen was used in the oxygenation experiments.

The results (Table II) indicate that pressure aeration will increase the ratio of AMC to 2,3-BG formed. When methylene blue was used in addition to pressure aeration, the values approached the AMC:2,3-BG

TABLE II  
*Effect of Pressure-Aeration on Production of Acetylmethylcarbinol*

Concentration methylene blue	Pressure	Acetylmethyl- carbinol	2,3-Butylene glycol	AMC+2,3-BG	AMC:2,3-BG
	<i>lbs.</i>	<i>mM</i>	<i>mM</i>	<i>mM</i>	
0	0	17.8	55.2	73.0	0.32
1/30,000	0	27.8	50.6	78.4	0.55
0	30	31.6	53.0	84.6	0.60
1/30,000	30	43.9	33.0	76.9	1.33
*1/30,000	30	44.6	27.2	71.8	1.64

Products in mM/100 mM glucose fermented.

Methylene blue added 72 hours after inoculation.

Time of fermentation: 96 hours.

\* Aeration with oxygen replacing air after 72 hours.

ratio obtained with "resting" *Aerobacter* cells. When the pressure-aerated fermentation to which methylene blue had been added was continued an additional 24 hours, the AMC: 2,3-BG ratio increased from 0.31 to 0.51. These increases were always found in aerated glucose fermentations as the fermentation time increased. As with the normally aerated fermentations, those to which methylene blue has been added at the time of inoculation had a lower AMC: 2,3-BG ratio than did the fermentations to which methylene blue was added 72 hours after inoculation (Table III). It appears that pressure aeration initiated toward the end of fermentation has little or no effect; pressure aeration

started when the methylene blue is added, 70 hours after inoculation, gave about the same AMC: 2,3-BG ratio as did a fermentation which was aerated under atmospheric pressure.

Ten l. of medium were added to a 20-l. carboy and inoculated in the usual manner. The flask was pressure aerated through five aerating stones. Methylene blue was added after 72 hours and the fermentation allowed to continue 34 hours longer. The pH after 106 hours was 5.9 and the glucose was completely fermented.

The AMC: 2,3-BG ratio was 0.78; 33.2 mM of AMC and 42.8 mM of 2,3-BG/100 mM of original glucose were obtained. A similar experi-

TABLE III  
*Relation Between Pressure-Aeration and Time of Inoculation*

Time of addition of methylene blue after inoculation	Pressure-aeration initiated after inoculation	Acetyl-methyl-carbinol	2,3-Butylene glycol	AMC+ 2,3-BG	AMC:2,3-BG
<i>Hrs.</i>	<i>Hrs.</i>	<i>mM</i>	<i>mM</i>	<i>mM</i>	
0	0	34.8	51.1	85.9	0.68
72	0	43.9	33.0	76.9	1.33
*0	70	24.9	48.7	73.6	0.51

Products in mM/100 mM glucose fermented.

Methylene blue, final concentration: 1/30,000.

Time of fermentation: 96 hours.

\* Fermentation time extended to 120 hours.

ment in which one aerating stone was used resulted in an AMC: 2,3-BG ratio of 0.27; 15.8 mM of AMC and 58.6 mM of 2,3-BG were produced/100 mM of glucose. These experiments indicate the importance of aeration in large-volume fermentations for the production of AMC.

### SUMMARY

The addition of methylene blue to fermentations of glucose by *Aerobacter aerogenes* increases the yields of acetylmethylcarbinol. The increase takes place at the expense of 2,3-butyleneglycol. The methylene blue poises the system at a redox potential where it may function as a hydrogen acceptor to bring about the oxidation of the glycol to the carbinol.



Oxygen or air under pressure also increases the conversion of 2,3-butylene glycol to acetylmethylcarbinol since it functions as a hydrogen acceptor. Use of both methylene blue and pressure was more effective than the use of either alone.

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# The Magnetic Properties of the Cobalt-Histidine Complex before and after Oxygenation<sup>1</sup>

L. Michaelis

*From the Laboratories of The Rockefeller Institute for Medical Research, New York*

Received February 7, 1947

The ability of hemoglobin and hemocyanine to reversibly combine with, or release, molecular oxygen, the equilibrium depending on the oxygen pressure, was until recently considered as quite unique. No model substance with similar properties, that might be prepared in the laboratory, had been known. It appeared especially remarkable that the ferrous state of the iron atom, or the cuprous state of the copper atom, was not oxidized to the ferric, or cupric state, respectively, after attachment of the oxygen molecule. Recently, however, metal complex compounds with properties more or less resembling those of hemoglobin have been discovered. The study of such compounds may eventually elucidate the structural conditions of a metal complex compound which endow it with the physiologically important property of reversible combination with molecular oxygen, or oxygenation, as Conant has designated this process, in distinction to oxidation.

The history of this field of research is quite recent and is as follows: In a paper on metal complex compounds by Pfeiffer and associates (1) it is mentioned that the salicylaldehyde-ethylenediimine complex of cobalt forms red-brown crystals which turn dark brown on exposure to the air. Pursuing this observation, Tsumaki (2) who had been a coworker on the paper just mentioned, later investigated this phenomenon more carefully and found that this discoloration is due to a reversible absorption of molecular oxygen. He compares this case with that of oxyhemoglobin. Pursuing this isolated observation, Calvin (3, 4) and his group discovered that a great number of chelated cobalt complex compounds of special structure can reversibly combine with molecular oxygen both in the solid, crystalline state and when dissolved in suitable organic solvents. The imitation of the naturally occurring oxygen carriers went a step further as Burk *et al.* (5) discovered that the cobaltous complex of histidine (1 Co:2 histidine) combines reversibly with molecular oxygen in *aqueous* solution. So far, all laboratory-made oxygen carriers have been cobalt compounds only. The conditions for making comparable iron or copper compounds have not yet been established.

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<sup>1</sup> Dedicated to Professor Carl Neuberg on his 70th birthday.

As regards hemoglobin, a remarkable change of the magnetic properties on oxygenation was discovered by Pauling and Coryell (6). Hemoglobin is paramagnetic, its dipole moment indicative of four unpaired electrons, just as in the free ferrous ion. Oxyhemoglobin, however, is diamagnetic, showing that not only have the four unpaired electrons of the ferrous ion been rearranged to form two pairs, but also the two unpaired electrons of the oxygen molecule have formed a pair. In oxyhemoglobin, the central iron atom has its electronic shells fully occupied to imitate the noble gas, krypton. It seemed worth while to investigate the magnetic susceptibility of the cobalt-histidine complex and its changes after oxygenation. The results of such an investigation are interesting for the study of the paramagnetism of complex compounds in general, and may even be correlated some day to the oxygen-carrying property of those complex compounds.

As regards the compounds described by Calvin and his group, the authors distinguish two classes. In one class, the paramagnetic susceptibility of the complex is indicative of one unpaired electron, and the compound becomes diamagnetic after oxygenation. In another class, there are originally three unpaired electrons, and the compound remains paramagnetic after oxygenation but only to the extent corresponding to one unpaired electron. In any case, oxygenation diminishes the paramagnetic susceptibility of the complex.

In this paper the magnetic behavior of the cobalt-histidine complex will be discussed. It represents still another type: here a complex with three unpaired electrons becomes diamagnetic on oxygenation.

The complex compounds are prepared by mixing stock solutions of cobaltous chloride ( $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ) and histidine (as hydrochloride +  $1 \text{ H}_2\text{O}$ ) in the ratio of 1 Co:2 histidine, under strictly anaerobic conditions. According to the amount of buffer or NaOH applied in addition, the complex can be obtained in various conditions of pH. At pH 6-7 its color is slightly pink, at pH 11, intensely purple, and in strong alkali it is blue. When a solution of the complex at any pH  $> 5$  is exposed to the air, the color turns intensely brown. The color can be reversed by pumping out the oxygen.<sup>2</sup>

The reversible uptake of oxygen comes to equilibrium in a few minutes. Thereafter, according to the authors mentioned, a secondary, very slow and irreversible reaction takes place, which is interpreted as the absorption of a second molecule of oxygen. Since it is reported to be irreversible, and its nature has not yet been fully studied, it will not be discussed here.

The reversibility of the first, fast reaction is in striking contrast to the behavior of similar reactions studied some years ago in this laboratory (7, 8) concerned with complexes of cobalt and iron with thioglycolic acid or cysteine. This difference is due to the fact that in our

<sup>2</sup> Since the brown color of the oxygenated complex is very intense, the reversibility can be best demonstrated by using a very dilute solution of the complex, say 0.001 M. When this solution, after turning brown, is pumped out in a Thunberg tube immersed in lukewarm water, the color fades out. It reappears after exposure to air.

previous studies the complexes were formed with readily oxidizable molecules, such as cysteine or thioglycolic acid, with the idea in mind of elucidating the role of complex formation in the catalytic autoxidation of the organic molecules. In the present case, however, we have to deal with an organic compound which is not as readily susceptible to oxidation.

The magnetic susceptibility of the complex compound in the dissolved state was measured with a modification of what is usually called Gouy's method as described previously (the "deflection method") (9, 10). The solutions, prepared anaerobically, were transferred into the cylindrical test vessel as rapidly as possible so that the degree of oxygenation was negligibly small. The results are shown in Table I.

TABLE I

Concentration of $\text{Co}^{++}$	pH (approximate)	Temperature of measurement	$\chi_{\text{mol}}$ , susceptibility in c.g.s. $\times 10^4$	$\epsilon_{\text{eff}}$ , effective magnetic moment
<i>g. at. / l.</i>		$^{\circ}\text{C.}$		<i>Bohr magnetons*</i>
0.05	5.5	23	+10,200	$\left. \begin{array}{c} 4.95 \\ 5.00 \\ 4.97 \\ 4.94 \end{array} \right\} \text{Average } 4.97$
0.05	5.5	21	10,620	
0.05	7.5	23	10,400	
0.10	8.5	23	10,200	
			Average 10,400	
0.05 (blue)	11.0	23	8,740	4.55

\* Calculated according to the formula  $\epsilon_{\text{eff}} = 2.84 \sqrt{\chi_{\text{mol}} \cdot T}$ , where  $T$  is the absolute temperature.

A solution containing the equivalent amount of NaCl instead of  $\text{CoCl}_2$  was chosen as reference for the susceptibility, so that the paramagnetic susceptibilities as observed need no correction for diamagnetism. The magnetic dipole moment, according to theory, due to electron spin alone is expected to be  $\sqrt{n(n+1)}$  Bohr magnetons for a molecule containing  $n$  unpaired electrons. This is 3.88 BM for three unpaired electrons and 4.89 for 4 unpaired electrons. In any ionic cobaltous compound there can be no more than three unpaired electrons ( $3d^7$ ), the excess of the observed value over the theoretical one may be attributed to orbital contributions. This excess (amounting to slightly more than one BM) is rather high, which is, however, usual for a cobaltous compound.

After the absorption of oxygen, the susceptibility always decreases, more or less according to the degree of saturation with oxygen. Even on measuring complexes possibly saturated with oxygen the values are not quite reproducible, since even slight differences in oxygen content will influence the result. However, those differences in the final results are quite irrelevant as to the interpretation of the results. So only one experiment will be described in detail among many others. A solution such as was used before, but after bubbling with pure oxygen for 10 minutes at room temperature, had a molar susceptibility of  $+120 \times 10^{-6}$ . If one tries to take this figure seriously and to convert it into Bohr magnetons the result would be 0.054 BM, which is meaningless. Undoubtedly, the small paramagnetic susceptibility observed is due to the fact that the oxygenation was not quite 100% and the oxygenated complex itself is diamagnetic. Other experiments gave similar but, for obvious reasons, not quite reproducible results. So we may take the original complex as an ionic complex, having the same susceptibility as the cobaltous ion itself, and the oxygenated one as a covalent one. Two of the unpaired electrons of the anaerobic complex, on oxygenation, form a pair, and the third forms a pair with the one unpaired electron of the oxygen molecule shared with another molecule of cobalt-histidine complex.

Hence, two quite different examples of reversible oxygenation are now known, where an originally paramagnetic metal complex becomes diamagnetic on oxygenation, and where originally ionic bonds change, on oxygenation, to covalent bonds. It is especially noteworthy that the oxygen molecule, which in the free state is paramagnetic, becomes diamagnetic when attached to the complex. There are two ways in which oxygen can be attached. In hemoglobin oxygen occupies one coordination place and thus raises the number of coordination places to the maximum, namely, six. (Four for the four nitrogen atoms of porphyrin, a fifth for the protein, and the sixth for oxygen.) Here it may be said that the oxygen molecule gives up its originally triplet state (or bi-radical state) with two unpaired electrons, and assumes a diamagnetic state which is not the ground state of the oxygen molecule in the free state. In cobalt-histidine the oxygen molecule forms a bridge between two molecules of the metal complex compound, making a binuclear complex. Each of the unpaired electrons of oxygen pairs with one unpaired electron of the cobaltous atom, which has three of them, and the other two form a pair in their turn. Ferrous ion has four

unpaired electrons, an even number. In order that combination of hemoglobin with oxygen should bring the electronic structure of iron to that of a noble gas structure (krypton), one single molecule of oxygen must contribute both its unpaired electrons to form a compound with all electrons paired. Cobalt in the cobaltous state has three unpaired electrons, an odd number. To make it even (a necessary condition for diamagnetism) it must accept only one electron from oxygen, and thus one oxygen molecule must be shared by two cobalt complex molecules.

Since the attachment of oxygen is reversible it does not seem appropriate to consider the oxygenated complex as a cobaltic compound. However, its magnetic property, *viz.*, its diamagnetism, is the one usually encountered in cobaltic complexes and is unique for this particular cobaltous compound.

In those two cases known in all details, where oxygen forms an oxygenated, not an oxidized complex, an originally paramagnetic molecule is converted after oxygenation to a diamagnetic one. One might wonder whether the property may be correlated with the fact that the oxygenation does not lead to oxidation. However, one must wait for more experimental data, especially also on hemocyanine, to arrive at generalizable conclusions, and must keep in mind that thus far all laboratory-made oxygen carriers are compounds of cobalt, not of iron or copper.

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# Bacterial Conversion of Pantothenic Acid into Coenzyme A (Acetylation) and its Relation to Pyruvic Oxidation<sup>1,2</sup>

G. David Novelli and Fritz Lipmann

*From the Biochemical Research Laboratory, Massachusetts General Hospital,  
Boston 14, Massachusetts*

Received March 17, 1947

## INTRODUCTION

During work on cell-free acetylation (1) the action of a new coenzyme was observed. More recently this coenzyme was identified as a pantothenic acid derivative (11) for which the term coenzyme A was introduced. The ubiquitous occurrence of coenzyme A and its content of pantothenic acid suggested at once a major role in metabolism. Its action in acetylation, although significant, appeared to be only a minor function. A fuller understanding of its metabolic action seemed to be approached through the finding that coenzyme A is a factor in a reaction of acetate with adenylypyrophosphate in liver, yielding a compound with the properties of acetyl phosphate (2). Preliminary data on this activation were already included in a recent review (3); more details will be published separately by Kaplan and Lipmann.

Further approaches to the study of the functioning of coenzyme A were opened through its characterization as a pantothenic acid derivative. Dorfman *et al.* (4) had shown with *Proteus morganii* that pantothenic acid was concerned with pyruvic acid oxidation. Their results were confirmed and amplified by Hills (5) (*cf.* also (6)). Particularly the experiments of Hills make it probable that the pantothenate effect applies to the second, or acetate, phase of pyruvate oxidation. He showed that in deficient organisms pyruvate may disappear completely, although at a slower rate than with added pantothenate. Per mole of pyruvate, however, only half a mole of oxygen was used and one mole

<sup>1</sup> Dedicated to Professor Carl Neuberg on his 70th birthday.

<sup>2</sup> Supported by a grant from the Commonwealth Fund.



of  $\text{CO}_2$  appeared. When pantothenate was added to the system the oxygen consumption rose to 2.5 moles/mole of pyruvate and the respiratory quotient became 1.25 instead of 2. In other words, without pantothenate, pyruvate oxidation stopped at acetate, while with pantothenate, the oxidation proceeded to completion. In addition to its pantothenic acid content, therefore, the effect of coenzyme A on phases of acetate metabolism in animal tissues suggested that coenzyme A may be the missing factor in the pantothenic acid-deficient organisms.

## MATERIALS AND METHODS

### *Microorganisms*

Two suitable strains of *Proteus morganii*, P-2 and P2818 were kindly supplied to us by Dr. McIlwain of the University of Sheffield, England. Deficient organisms were grown on Fildes' medium (7) modified by using the salt solution described by Snell *et al.* (8). The lactate was replaced with glucose and 25 mg. cystine and 1.25 mg. nicotinamide/l. were added. To obtain reasonable yields 4–8  $\gamma$  pantothenic acid/l. had to be supplied.

*Lactobacillus arabinosus* 17-5 was kindly supplied by Henry Kamin of Duke University. The organism was recommended for pantothenic acid assay by Skeggs and Wright (9) and has been used by us for this purpose throughout work on coenzyme A (11). The medium of Cheldelin *et al.* (10) was used to grow deficient organisms.

### *Coenzyme Determination*

The test method used was the enzymatic acetylation of sulfanilamide in pigeon liver extract (1). The apoenzyme is prepared by aging a 10% solution of acetone powder of pigeon liver for four hours at room temperature. In one ml. total volume, 0.25 ml. liver extract, 8  $\mu\text{M}$  ATP, 10  $\mu\text{M}$  cysteine, bicarbonate, sulfanilamide and acetate and various amounts of coenzyme are present. A standard curve is obtained of the type reproduced in the paper of Lipmann (3). A unit of coenzyme is that amount which gives half activation. Two points are determined near the mid-point for every sample and the unit value is read from the standard curve.

A detailed account of the quantitative determination of coenzyme A will be given in a subsequent communication by Kaplan and Lipmann.

*Manometric experiments* were carried out in the usual manner. Warburg vessel with annexes and average volumes of 9 ml. or of 18 ml. were used.

## SYNTHESIS OF COENZYME A FROM PANTOTHENIC ACID

Two types of experiments were carried out: (1) growth experiments with *L. arabinosus* in graded amounts of pantothenic acid and (2) short term experiments with non-growing pantothenate-deficient *Proteus morganii*.

(1) *L. arabinosus*, as used for pantothenic acid assay, was inoculated into 100 ml. of medium containing 1, 50, 100, and 500 $\gamma$  of pantothenic acid. After 56 hours the organisms were collected by centrifuging, washed, and made up to 2 ml. The suspension was autoclaved (120°C., 15 lbs. pressure) for 5 minutes. Coenzyme was determined in the supernatant of the autoclaved suspension. In this manner the data listed in Table I were obtained.

TABLE I  
*Coenzyme Synthesis in L. arabinosus*

Pantothenic acid was determined microbiologically. Coenzyme was determined enzymatically.

No.	Pantothenic acid added to growth medium	Per g. dry weight of harvested cells				
		Units coenzyme	Pantothenic acid bound in coenzyme, calculated**	Yield of coenzyme	Free pantothenic acid	Bound* pantothenic acid
1	$\gamma/100\text{ ml.}$			<i>per cent</i>		
	1	0***	—	—		
	50	44	31	4.8		
	100	81	57	4.2		
	500	89	62	1.0		
2	30	54	38	7.8	7	33
	75	94	66	4.8	7	51

\* Bound pantothenic acid is determined after liberation through incubation with a mixture of acetone pigeon liver extract and intestinal phosphatase. This enzyme mixture was shown to liberate pantothenic acid from coenzyme A (11). Intact coenzyme does not test as pantothenic acid microbiologically.

\*\* This value is calculated by multiplying the number of coenzyme units with the constant factor of 0.7 $\gamma$  pantothenic acid/unit coenzyme (11).

\*\*\* Too little to be determined accurately.

With 0.01 $\gamma$  pantothenic acid/ml. no determinable amounts of coenzyme were formed. With 0.5 and 1.0 $\gamma$ /ml. the coenzyme formation was proportional to pantothenic acid concentration. Above 1.0 $\gamma$  pantothenic acid/ml. little increase in coenzyme formation occurred. As shown elsewhere (11) a unit coenzyme represents 0.7 $\gamma$  of pantothenic acid. Using this factor, the yield of coenzyme amounts to 4-8% in the region of proportionality. As a check on the proportionality between bound pantothenic acid and coenzyme, in one experiment free and

"bound" pantothenic acid were determined microbiologically in addition to coenzyme A. To make the pantothenic acid, bound in the coenzyme, available to the microorganism the coenzyme was decomposed by the special enzyme treatment described previously (11). The good agreement between bound pantothenic acid as determined and as calculated may serve as a check on the nature and pantothenic acid content of the coenzyme (Table I).

(2) The previously mentioned experiments of Dorfman *et al.* (4) and of Hills (5) had shown that the pyruvic oxidation of pantothenic acid-deficient *Proteus morganii* was stimulated by addition of pantothenic acid. If such a stimulation was attributable to a formation of coenzyme A, an increase in coenzyme under such conditions was to be expected.

*Proteus morganii* was grown in a deficient medium containing 8 $\gamma$  of pantothenic acid/l. The organisms were harvested after 18 hours. A longer growth period did not materially improve returns. The yield was 15–20 mg. dry weight/l. The cells were centrifuged and washed and a suspension made up in 0.02 *M* phosphate (pH 7) containing 10–20 mg. of dry weight/ml. Of this suspension 0.5 ml. generally was used per Warburg vessel. The final volume was 1.4 ml.

TABLE II

*Effect of Pantothenic Acid on Pyruvate Respiration of Resting,  
Pantothenic Acid-Deficient P. morganii*

Pantothenic acid added	Q <sub>O<sub>2</sub></sub> (0–60')	
	30 $\gamma$	None
P-2	9.9	6.4
P-2818	12.8	9.1
P-2818	10.2	5.9

In this manner the data of Table II were obtained. The samples with and without pantothenic acid were equilibrated for 30 minutes at 37°C. before pyruvate was dipped into the main chamber. With added pantothenic acid increases of 40–70% were obtained in confirmation of the earlier mentioned work of Dorfman and of Hills.

In parallel with the last respiration experiment of Table II, samples of the same batch of organisms were shaken for 3 hours with pyruvate with and without addition of pantothenate. At the end of the period the suspension was autoclaved and analyzed for coenzyme. In Table III Q<sub>O<sub>2</sub></sub> and coenzyme values are compared.

TABLE III  
*Comparison of the Effect of Added Pantothenic Acid on  
Respiration and on Coenzyme A Content*

	Q <sub>O<sub>2</sub></sub>	Coenzyme units per g. dry weight
+ Pyruvate	5.9	130
+ Pyruvate and pantothenate	10.2	520

The results show the expected parallel between increase of respiration and coenzyme values when pantothenic acid was added.

### SUMMARY

A conversion of pantothenic acid into coenzyme A is shown with *L. arabinosus* and *Proteus morganii*. The effect of pantothenic acid on pyruvate oxidation of deficient *Proteus morganii* (Dorfman, Hills) is confirmed. A four-fold increase in coenzyme A concentration was found to parallel the respiratory stimulation by pantothenate.

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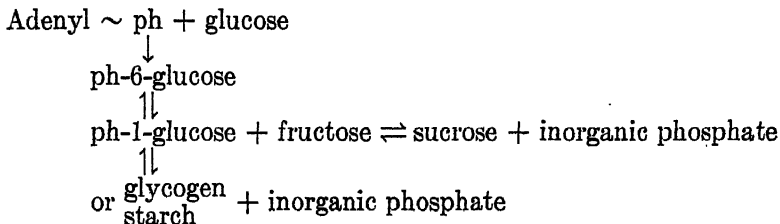
# Enzymatically Synthesized Disaccharides<sup>1</sup>

W. Z. Hassid, M. Doudoroff and H. A. Barker

*From the Division of Plant Nutrition, College of Agriculture, and the  
Department of Bacteriology, University of California, Berkeley*

Received February 10, 1947

The bacterium *Pseudomonas saccharophila* contains a phosphorylase which catalyzes the reversible reaction: sucrose + inorganic phosphate  $\rightleftharpoons$  glucose-1-phosphate + fructose (1, 2). Crystalline sucrose was thus prepared from glucose-1-phosphate and fructose and shown to be identical with natural sucrose (3). The mechanism of sucrose breakdown and formation is analogous to that of starch and glycogen (4, 5). After glucose is combined with inorganic phosphate to form  $\alpha$ -glucose-1-phosphate, the ester bond of this hexosephosphate is exchanged for a glycosidic bond when it combines with fructose, thus forming sucrose. This may occur by the following series of reactions:



The first step, the conversion of the energy-rich  $\sim \text{ph}$  into the ordinary ester-ph, is accomplished by a loss of 8,000 calories, and is, therefore, essentially irreversible (6). The subsequent steps are all readily reversible, involving only small energy changes. The reaction requiring the most energy is the phosphorylation of glucose to glucose-1-phosphate. When the latter is formed it is transformed into disaccharide or polysaccharide practically without any expenditure of energy.

As in the phosphorolysis of starch and glycogen, the formation of glucose-1-phosphate and fructose from sucrose and inorganic phosphate

<sup>1</sup> Dedicated to Professor Carl Neuberg on his 70th birthday.

may be considered to occur as the result of phosphorolytic cleavage of glucose from the sucrose molecule, the sucrose being disrupted without water entering into the reaction. The reverse reaction, the formation of sucrose from glucose-1-phosphate and fructose is the result of "de-phosphorolytic" condensation of the two monosaccharides. The reversible phosphorolysis of sucrose can be represented as shown in Fig. 1.

Owing to the successful *in vitro* synthesis of sucrose we have gained further knowledge of the chemical constitution of this carbohydrate. The type of glycosidic linkages which combine glucose and fructose in the sucrose molecule has hitherto not been known with certainty. Although the work of the late E. F. Armstrong (7) and that of Purves and Hudson (8) indicate that the structure of sucrose is  $\alpha$ -D-glucopyranose- $\beta$ -D-fructofuranoside, it still remained an open question as to

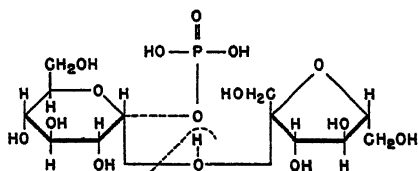


FIG. 1. Phosphorolysis of Sucrose, Showing the Entrance of Inorganic Phosphate at the Glucosidic Linkage.

whether the  $\alpha$ - or  $\beta$ -form of either hexose was involved in joining the monosaccharide units to form the disaccharide, sucrose. By showing that naturally occurring sucrose is identical with the enzymatically produced product and that it is synthesized through "de-phosphorolytic" condensation of the  $\alpha$ -form of glucose-1-phosphate and fructose, and  $\alpha$ -configuration of glucose in the sucrose molecule has been confirmed. Evidence regarding this type of linkage is adduced from the observation that whenever the  $\alpha$ -linkage of the phosphoric acid in glucose-1-phosphate is exchanged for a glycosidic linkage with another monosaccharide the type of linkage is not altered. This is exemplified by the formation of starch and glycogen from glucose-1-phosphate (4, 5).

The enzymatic synthesis of sucrose also throws light on the formation of the furanose form of fructose in the sucrose molecule. The fact that sucrose is directly formed from glucose-1-phosphate and fructose supports the view that the latter monosaccharide occurs in solution in an equilibrium mixture of furanose and pyranose forms. This makes it







unnecessary to postulate a special mechanism of stabilization of a five-membered ring before the formation of compound sugars containing the fructose molecule (9).

Various attempts to synthesize sucrose chemically, by condensation of tetraacetylfructofuranose with tetraacetylglucopyranose in the presence of a dehydrating agent, have failed. Irvine *et al.* (10, 11) in attempting to combine these acetylated glucose and fructose derivatives with the expectation of producing sucrose octaacetate obtained a disaccharide derivative with a different glycosidic linkage than sucrose, the so-called *iso*-sucrose.

It may be pointed out that even had such chemical synthesis of sucrose been achieved it would not have given us any information as to how this compound is synthesized in living organisms, because it is well established that syntheses of organic compounds in living cells often take place through *entirely different* mechanisms than in the test tube. For this reason the discovery of the enzymatic synthesis of sucrose is perhaps of greater interest than its chemical synthesis, at least to the biochemist.

In considering the process of glycogen and starch synthesis, it is observed that these polysaccharides are formed by a general mechanism which operates in various living cells. Thus, regardless of the source of the enzyme phosphorylase, whether it is derived from animal, yeast or plant cells, it will act on glucose-1-phosphate to form essentially a similar polysaccharide. It can therefore be assumed that, except for minor variations, the mechanism for glycogen and starch formation is similar in all living cells. This cannot be said with regard to the process of sucrose formation. The mechanism for sucrose synthesis in which the enzyme system derived from the bacterial organism, *Ps. saccharophila* is involved does not appear to apply to higher plants.

A similar enzyme system that would combine glucose-1-phosphate and fructose to form sucrose and inorganic phosphate has not yet been isolated from the tissues of higher plants, despite various attempts to do so. However, biochemical studies on various species of plants support the view that the synthesis of sucrose does proceed by chemical reactions in which glucose or fructose phosphate esters, or both, serve as substrates, although the mechanism is probably not identical with that of the bacterial enzyme system. It is also significant that the experimental evidence now available shows that, for the synthesis of sucrose from glucose and fructose in the plant, aerobic metabolism is

indispensable (12, 13). Possibly aerobic oxidations are essential to the phosphorylation of one of the substrates involved in the synthesis of sucrose.

### *Specificity of Sucrose Phosphorylase*

Like potato and muscle phosphorylase from *Ps. saccharophila*, the enzyme is specific with regard to glucose-1-phosphate. Potato or muscle phosphorylase will not form polysaccharide from  $\alpha$ -maltose-1-phosphate,  $\alpha$ -galactose-1-phosphate,  $\alpha$ -mannose-1-phosphate, or  $\alpha$ -xylose-1-phosphate (14). Similarly, these hexosephosphates cannot be substituted for glucose-1-phosphate when use is made of bacterial sucrose phosphorylase, which has the ability to combine glucose-1-phosphate with fructose to form sucrose. This enzyme is, however, quite versatile with regard to substituents for the second sucrose component, fructose. The sucrose phosphorylase will combine the same ester with other ketose monosaccharides, such as D-ketoxylase and L-sorbose to form the corresponding non-reducing disaccharides, the D-glucosido-D-ketoxylase and the D-glucosido-L-sorbose. The same enzyme will also cause glucose-1-phosphate to react with the aldopentose, L-arabinose, forming a reducing disaccharide, D-glucosido-L-arabinose.

### *D-Glucosido-D-ketoxylase and D-Glucosido-L-sorbose*

The enzymatically synthesized D-glucosido-D-ketoxylase (15) and the D-glucosido-L-sorbose (16) are non-reducing, showing that, as in sucrose, the glucose and ketose monosaccharides are linked through the carbonyl groups. In proving the structure of these disaccharides, the technique of Hudson and coworkers (17) for oxidizing carbohydrates with sodium periodate was used. In a disaccharide consisting of glucopyranose and ketofuranose united through positions 1 and 2, the glucose residue would possess three adjacent free hydroxyls, on carbon atoms 2, 3 and 4, and the ketose residue would possess two free hydroxyls, on carbon atoms 3 and 4. On oxidation of such a disaccharide with periodate a total of 3 moles of periodate would be consumed and one mole of formic acid would be formed per mole of disaccharide. Actually, both disaccharides gave experimental data which agreed with this expectation. Any other ring structure for either the glucose or the ketose component would have given divergent data.

The fact that these disaccharides, like sucrose, are formed as a result of "dephosphorolytic" condensation involving  $\alpha$ -D-glucose-1-phosphate supports the view that glucose also exists in the disaccharides as the  $\alpha$ -form.

The disaccharides give a blue-green color with diazouracil, a reaction shown by Raybin (18) to be specific for sucrose or any other compound containing the same type of glycosidic glucose-fructose linkages that exist in sucrose, such as raffinose, gentianose, and stachyose.

The fact that the phosphorylase from *Ps. saccharophila*, which can effect the synthesis of sucrose from glucose-1-phosphate and D-fructose, can also effect the synthesis of disaccharides from glucose-1-phosphate and L-sorbose or D-ketoxyllose, strongly suggests that the local structure existing where the linkage occurs between the two monosaccharide units in the two disaccharides is the same as it is in sucrose. The observation that the glucosidosorbose and the glucosidoketoxylside disaccharides give the Raybin color reaction, which has so far been found to be positive only for carbohydrates containing the sucrose linkage, adds force to the suggestion that the new disaccharides have the same linkage that exists in sucrose.

Since the structure of sucrose is regarded as being  $\alpha$ -D-glucopyranosido- $\beta$ -D-fructofuranoside, there is good reason to believe that the linkage involved in the new disaccharides is of the same type, that is, glucose exists as the  $\alpha$ - and fructose as the  $\beta$ -form in this disaccharide. The above evidence and analogy with sucrose indicate that the structure of the glucosidoketoxylside is  $\alpha$ -D-glucopyranosido- $\beta$ -ketoxylfuranoside as shown in Fig. 2. This compound and sucrose appear to be

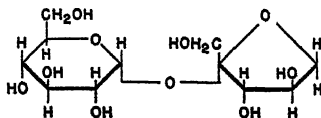


FIG. 2.  $\alpha$ -D-Glucopyranosido- $\beta$ -D-ketoxylfuranoside

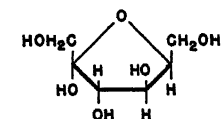
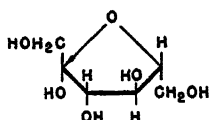
structurally identical, except that sucrose has an additional carbon atom with a primary alcohol attached to the ring.

With regard to the linkage of the D-glucosido-L-sorbose the situation is somewhat different. The sorbose unit in this disaccharide is an L-sugar in contrast to the D-fructose unit existing in sucrose. Since  $\beta$ -D-fructose and  $\alpha$ -L-sorbose have the same configuration for their second carbon atoms (Fig. 3) it is necessary to designate the ketose portion of this disaccharide as  $\alpha$ -L-sorbose. The structural formula for this disaccharide is represented by Fig. 4.

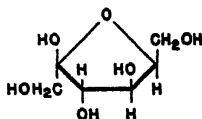
It is noteworthy that fructose, which has a pyranose structure when existing in the free state, assumes a furanose configuration whenever it combines with another sugar to form an oligosaccharide or polysaccharide. Apparently, the ketohexose, L-sorbose, shows the same behavior.

A third non-reducing disaccharide, consisting of D-glucose and L-ketoarabinose has recently been synthesized by the same enzyme obtained from *Ps. saccharophila*. The constitution of this carbohydrate is at present under investigation.

$\beta$ -D-Fructofuranose



$\alpha$ -L-Sorbofuranose



$\beta$ -L-Sorbofuranose

FIG. 3.

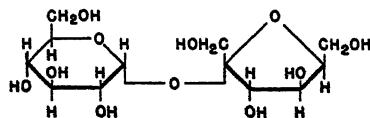


FIG. 4.  $\alpha$ -D-Glucopyranosido- $\alpha$ -L-sorbofuranoside

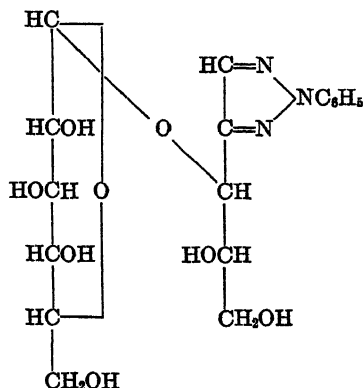
### D-Glucosido-L-arabinose<sup>2</sup>

This disaccharide reduces Fehling and alkaline ferricyanide solutions. Its specific rotation  $[\alpha]_D$  in water is  $+156^\circ$ . On hydrolysis with acid it produces one mole of D-glucose and one more of L-arabinose. The phenylosotriazole derivative of the disaccharide prepared according to Hudson *et al.* (19) is readily hydrolyzed with acid to D-glucose and L-arabinose phenylosotriazole showing that the L-arabinose constitutes the free reducing unit in the disaccharide. Like the previously isolated disaccharides, it is formed by the agency of phosphorylase from *Ps. saccharophila* as a result of "dephosphorylative" condensation involving  $\alpha$ -D-glucose-1-phosphate, indicating that glucose exists in the disaccharide as the  $\alpha$ -form.

On oxidation of the phenylosotriazole derivative of the disaccharide with sodium periodate, three moles of periodate are consumed with the formation of one mole each of formic acid and formaldehyde per mole of phenylosotriazole derivative. The structure of this compound is, therefore, 3-[ $\alpha$ -D-glucopyranosido]-L-arabinose phenylosotriazole in

<sup>2</sup> Details of the structural study of this disaccharide will be published elsewhere.

which D-glucose is attached through carbon atom 1 to carbon atom 3 or L-arabinose as shown by Formula (I).



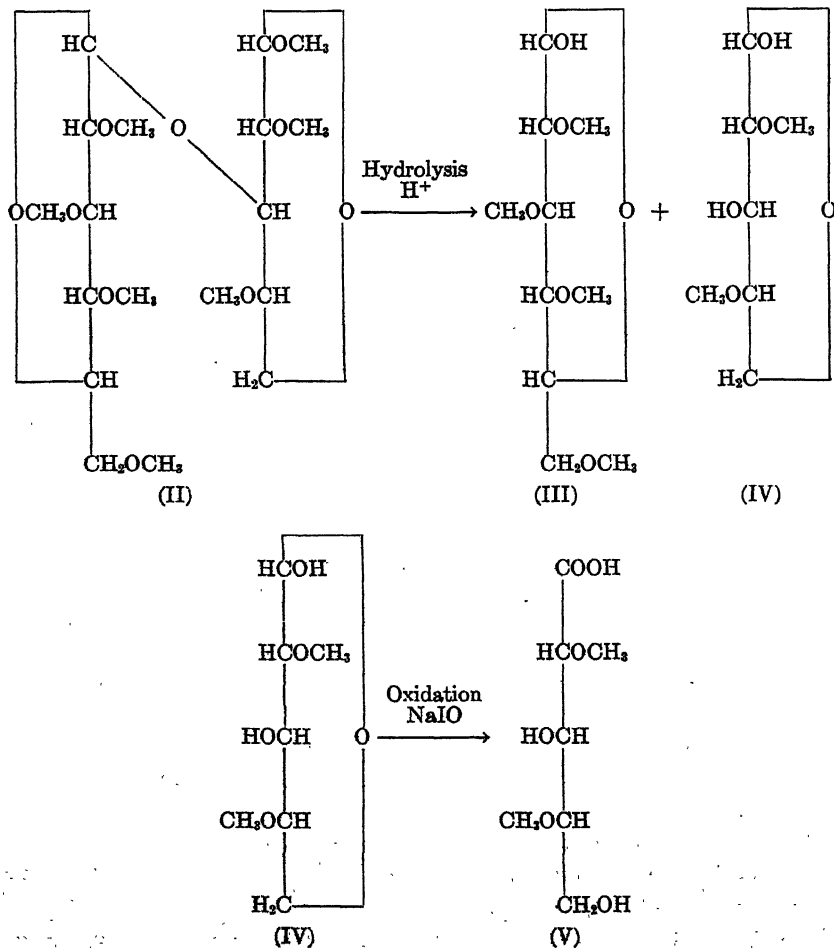
I. 3-[α-D-Glucopyranosido]-L-arabinose Phenylsotriazole

If the D-glucose in the D-glucopyranoside-L-arabinose phenylsotriazole were attached to carbon atom 4 of the L-arabinose derivative, oxidation of this compound with sodium periodate would require two moles of periodate with liberation of one mole of formic acid and no formaldehyde production. Junction of D-glucose to carbon atom 5 of the L-arabinose phenylsotriazole would require three moles of periodate, whereby one mole of formic acid would be produced in the reaction, but no formaldehyde formed.

On methylation of the disaccharide with dimethyl sulfate and sodium hydroxide a hexamethylmethyl derivative of the carbohydrate was obtained. When this fully methylated derivative (II) was hydrolyzed with acid, 2,3,4,6-tetramethyl-D-glucose (III) and dimethyl-L-arabinose (IV) was produced. Since position 3 in the L-arabinose component (I) was shown to be occupied in glycosidic linkage with D-glucose, the dimethyl-L-arabinose could be either the 2,5- or 2,4-dimethyl derivative (IV), depending on whether the L-arabinose unit originally exists in the disaccharide in the furanose or pyranose form. The ring type of the L-arabinose was ascertained by subjecting the dimethyl-L-arabinose to oxidation with sodium periodate, after it had been oxidized with hypiodite to the corresponding straight chain, dimethyl-L-arabonic acid (V).

If the dimethyl derivative were the 2,5-dimethyl-L-arabonic acid, it would possess a pair of adjacent hydroxyls, on positions 3 and 4, which, on oxidation with sodium periodate, would consume one mole

of periodate in the reaction. On the other hand, the 2,4-dimethyl-L-arabonic acid (V) lacking a pair of adjacent hydroxyls will not be oxidized. Actually, no periodate was consumed when the dimethyl-L-arabonic acid was treated with this reagent. This shows that the dimethyl derivative is 2,4-dimethyl-L-arabonic acid. The free hydroxyl in position 3 is obviously restored in the dimethyl-L-arabinose when the methylated disaccharide is hydrolyzed; the hydroxyl in position 5 is formed when its internal ring is broken in the process of oxidation to dimethyl-L-arabonic acid, which is a straight chain compound.



On the basis of these results the structural formula for this reducing disaccharide may be written as in Fig. 5.

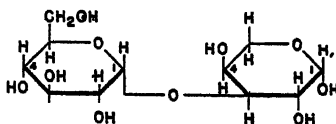


FIG. 5. 3-[ $\alpha$ -D-glucopyranosido]-L-arabinopyranose

#### ACKNOWLEDGMENT

The writers wish to express their thanks to the Corn Industries Research Foundation for its grant which helped to support the work reported in this paper and to Mr. W. H. Dore for his helpful criticism.

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# Investigations on the Acetone-Butyl Alcohol Fermentation<sup>1</sup>

Ernst Simon

*From the Daniel Sieff Research Institute, Rehovoth, Palestine*

Received January 9, 1947

## INTRODUCTION

Whereas the mechanism of carbohydrate breakdown in alcoholic fermentation and in glycolysis has been clarified both theoretically and experimentally, very little is definitely known on the mechanism of acetone-butyl alcohol fermentation. It is indicative of the situation that the most current assumption, *viz.*, the formation of acetone through the intermediate of acetic acid, has been supplemented by the recent work of Wood *et al.* (1) and of Wood, Brown and Werkman (2), who found, using labeled acetic acid, that 50% of this acid is converted into butyl alcohol by *Clostridium acetobutylicum*.

In continuation of previous experiments (3), the present paper deals with another approach to the elucidation of the mechanism of the acetone-butyl alcohol fermentation, *viz.*, the study of the relation between substrate structure and fermentability and, in particular, the structural features of the substrate which govern the fermentative formation of 4-carbon compounds. The results, summarized in a preliminary note (4), show the existence of certain fundamental differences between the enzyme systems present in yeast and animal tissues and those of the butyric organisms. While it is not yet possible to formulate an adequate sequence of reactions to describe such fermentations, two points have become clear:

1. The 3-carbon compounds, which are intermediates in all other fermentations, do not yield 4-carbon compounds under the experimental conditions employed in this investigation. Even pyruvic acid, which was said (5, 6) to form butyric acid, has only given us acetic acid, in accordance with results reported by Koepsell and co-workers (7).

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<sup>1</sup> Dedicated to Professor Carl Neuberg on his 70th birthday.

2. Pentoses, which are not attacked by yeast, are fermented to the same 4-carbon compounds and with the same yields as hexoses. It may be recalled that also another group of microorganisms (*Aerobacter*, *Aeromonas*, *Aerobacillus*) (8) converts pentoses and hexoses alike into a 4-carbon compound, *viz.*, 2,3-butylene glycol.

Whether pentoses are fermented and degraded by way of phosphorylated intermediates is still an open question. In the case of *Fusaria*, however, this does not seem to be the case (29).

From the two possible ways of obtaining information about production of 4-carbon compounds by fermentation, the determination of butyric acid as a criterion has been chosen in preference to the determination of butyl alcohol. Since the primary purpose of this study was aimed at the establishment of a functional relationship between the structure of the substrate and the formation of 4-carbon compounds *in general*, it is irrelevant what share 4-carbon compounds, if formed at all, may claim in the total balance.

## EXPERIMENTAL

### General

I. *Fresh Bacteria*. The experiments were carried out with a strain of *Cl. acetobutylicum* (Weizmann). Spore suspensions from fermented maize tubes were inoculated into 5% maize mash, incubated for 24 hours at 37°C. and then inoculated into 1% maize mash, containing 0.1% yeast extract. Five l. of 1% maize mash were inoculated with 8% of this fermenting mash after 6 hours and incubated for 18 hours at 37°C. Vigorous fermentation took place. The bacteria were then centrifuged off, washed 3 times with physiological salt solution and suspended in phosphate buffer (pH 6.2). Such a suspension did not attack glucose appreciably in the presence of *M*/15 phosphate buffer within 5 hours, but after 24 hours the substrate had completely disappeared and had formed the normal fermentation products.

II. *Acetone-Dried Bacteria*. Many of the experiments were carried out with acetone-dried bacteria. In those cases in which the preparations were compared with suspensions of fresh bacteria, no significant differences in the mode of action were detected.

In an iron vessel, 22.5 l. of 1% maize mash, containing 0.1% yeast autolyzate, were inoculated with 1.5 l. of fermenting 1% maize mash and incubated at 37°C. After 24 hours, the bacteria were centrifuged off in an overflow-centrifuge. The residue was thoroughly triturated with about 20 l. of physiological salt solution and centrifuged again. The bacteria were then treated successively with 1 l. of acetone for 8 minutes, with 450 ml. of acetone for 4 minutes, and with 500 ml. of ether for 3 minutes. Twenty-five g. of acetone-dried bacteria were thus obtained. Their activity was found to remain unimpaired for at least one year.

III. *Experimental Conditions*. The experiments were generally carried out with 180 mg. of glucose or equivalent amounts of the other substrates in phosphate buffer. At the end of an experiment, two-thirds of the mash were distilled off in presence of

phosphoric or sulfuric acid, and, in the distillate, the total amount of volatile acids and, by the Duclaux method, the ratio butyric/acetic acid was determined.

No attempt has been made thus far to determine quantitatively the non-acidic products, as the ratio of "neutral solvents" to acids is generally constant in the acetone-butyl alcohol fermentation.

#### IV. Preparation of Substrates.

1. *Hexose-6-monophosphate* (Neuberg-ester): from hexose-1, 6-diphosphate by acid hydrolysis (9).

2. *D-Arabitol*: by catalytic hydrogenation of D-arabinose (10).

3. *L-Arabonic acid* and *D-arabonic acid*: from L- and D-arabinose, respectively, by bromine oxidation (11).

4. *3-Phosphoglyceric acid* (barium salt): (12).

5. *Pyruvic acid*: from tartaric acid and potassium hydrogen sulfate (Organic Syntheses, Coll. Vol. 1, p. 462).

6. *Glycolaldehyde*: from dioxymaleic acid (13, 13a).

7. *Diacetoneglucose*: (14).

8. *Monoacetoneglucose*: from diacetoneglucose (15).

The other substrates used were commercial preparations.

### SYSTEMATIC EXPERIMENTS

The experiments reported here are listed according to the carbon number of the substrates, in decreasing order.

I. *7-Carbon-Compounds*. Perseitol,  $C_7H_{16}O_7$ , was not attacked by the bacteria.

II. *6-Carbon Compounds*.

1. To elucidate the possible influence of the grouping at the first C-atom on the formation of 4-carbon compounds, the following glucose derivatives were chosen as substrates: (1) glucose (aldehyde group), (b) mannitol, dulcitol, sorbitol (hydroxyl group), (c) gluconic acid (carboxyl group), (d) hexose-1-monophosphate (Cori-ester) (phosphorylated hydroxyl group).

*Composition of the Fermentation Mixtures*: (1) 10.0 ml. 3.12% glucose, 20.0 ml. M/15 phosphate buffer (pH 6.2), 20.0 ml. bacterial suspension, 50.0 ml. water. (2) 10.0 ml. 1.8% glucose, 15.0 ml. M/15 phosphate buffer, 50.0 ml. water, 0.3 g. acetone-dried bacteria.

*Controls*: (1) 20.0 ml. phosphate buffer, 20.0 ml. bacterial suspension, 60.0 ml. water. (2) 15 ml. M/15 phosphate, 60.0 ml. water, 0.3 g. acetone-dried bacteria.

*Result*: The formation of 4-carbon compounds is independent of the grouping at the first C-atom of the molecule: *all* substrates examined gave positive results. The ratio of butyric acid and acetic acid, however, varies—a point which will be discussed further below.

2. The importance of the terminal alcoholic group at  $C_6$  was then investigated.

(a) D-galacturonic acid (carboxyl group), (b) hexose-6-monophosphate (Neuberg-ester; phosphorylated hydroxyl group), (c) saccharic acid (carboxyl group at  $C_1$  and  $C_6$ ), (d) hexose-1,6-diphosphate (Harden-Young ester, phosphorylated hydroxyl group at  $C_1$  and  $C_6$ ).

TABLE I

Substrate	Bacterial preparation	Ratio butyric/acetic acid
D-Glucose	Fresh bacteria	1.6:1; 1:1
D-Glucose	Acetone-dried bacteria	1.6:1; 1:1
D-Mannitol	Acetone-dried bacteria	4:1
Dulcitol		4:1
D-Sorbitol		3:1
D-Gluconic acid (Sodium salt)	Fresh bacteria	1:3
	Acetone-dried bacteria	1:5
Hexose-1-monophosphate (Coi-ester)	Fresh bacteria	1:2.5
Hexose-1-monophosphate (Coi-ester)	Acetone-dried bacteria	1:2.5

TABLE II

Substrate	Bacterial preparation	Ratio butyric/acetic acid
D-Galacturonic acid (sodium salt)	Acetone-dried bacteria	Pure acetic acid
Hexose-6-monophosphate	Acetone-dried bacteria	1:10
Saccharic acid	Acetone-dried bacteria	1:4
Hexose-1,6-diphosphate	Acetone-dried bacteria	Methylglyoxal, traces of pyruvic acid

*Result:* An hydroxyl group at C<sub>5</sub> seems to be essential, since the formation of 4-carbon compounds is either completely suppressed (galacturonic acid, hexose-diphosphate) or at least diminished very substantially if that hydroxyl group is modified.

3. The fermentability of the aldoses had been previously established. As an additional example, a ketose, L-sorbose, was investigated. In contradistinction to the negative results of Underkofler and Hunter, Jr. (16), L-sorbose fermented like glucose; with acetone-dried bacteria the ratio butyric/acetic acid was 1.5:1.

4. Inositol, monoacetoneglucose and diacetoneglucose were not attacked by the bacteria. For the two acetone compounds, the same observation has been made for yeast.

*III. 5-Carbon Compounds.* The substrates examined were: L-arabinose, D-arabinose, D-xylose, D-arabitol, L-arabonic acid, D-arabonic acid, and L-rhamnose.

TABLE III

Substrate	Bacterial preparation	Ratio butyric/acetic acid
L-Arabinose	Fresh bacteria	1:2.5
	Acetone-dried bacteria	1:2.5
D-Arabinose	Acetone-dried bacteria	Not attacked
D-Xylose	Fresh bacteria	1:2.5
	Acetone-dried bacteria	1:1.6
D-Arabitol	Acetone-dried bacteria	2.5:1
L-Arabonic acid	Acetone-dried bacteria	Not attacked
D-Arabonic acid	Acetone-dried bacteria	Not attacked
L-Rhamnose	Acetone-dried bacteria	1:3

*Result:* The natural C<sub>5</sub>-sugars examined (L-arabinose, D-xylose, L-rhamnose) are all fermented; the negative result with D-arabinose indicates a stereochemical specificity. Oxidation of the aldehyde group to carboxyl abolishes the fermentability while its reduction to hydroxyl appears to be without deleterious influence.

*IV. 4-Carbon Compounds.* DL-Erythritol was not attacked by the bacteria.

*V. 3-Carbon Compounds.* The following compounds were used as substrates: (1) pyruvic acid, (2) 3-phosphoglyceric acid, (3) 3-phosphoglyceric acid + glycerophosphoric acid, (4) DL-glyceric aldehyde, (5) glycerol.

*Result:* 3-Phosphoglyceric acid, its equimolecular mixture with glycerophosphoric acid, and DL-glyceric aldehyde were not attacked

TABLE IV

Substrate	Bacterial preparation	Fermentation products formed
Pyruvic acid	Fresh bacteria	Pure acetic acid
	Acetone-dried bacteria	Pure acetic acid
3-Phosphoglyceric acid	Fresh bacteria	Not attacked
3-Phosphoglyceric acid + Glycerophosphoric acid DL-Glyceric aldehyde	Fresh bacteria	Not attacked
	Acetone-dried bacteria	Not attacked
Glycerol	Acetone-dried bacteria	Ratio butyric/acetic acid 2.5:1

by the bacteria. The latter substrate even inhibited the fermentation of glucose. Glycerol, on the other hand, ferments normally and from the butyric/acetic ratio must be classified with the pentitols and hexitols. Still more surprising is the behavior of pyruvic acid, which gives only acetic acid. This has been verified in a series of further experiments.

TABLE V

Bacterial preparation	Experimental conditions	Fermentation products formed
Fresh bacteria	Neutralized pyruvic acid solution, phosphate buffer (pH 6.2)	Pure acetic acid in quantitative yield
Fresh bacteria	Addition of $\text{CaCl}_2$ solution and physiological NaCl solution instead of phosphate buffer	
Acetone-dried bacteria	Phosphate buffer (pH 6.2)	
Acetone-dried bacteria	Phosphate buffer (pH 4.8)	

The same behavior is exhibited in the fermentation of mixtures of glucose and pyruvic acid.

TABLE VI

*Fermentation Experiments with a Mixture of Glucose and Pyruvic Acid*

Substrate:	355.1 mg. glucose	169.2 mg. glucose 186.4 mg. pyruvic acid	367.4 mg. pyruvic acid
Volatile acids (ml. N 10)	16.54	29.50	44.96
Ratio butyric/acetic acid	1:1	1:6	Pure acetic acid
Butyric acid, mg.	72.78	37.08	None

*Result:* Even in the presence of glucose, pyruvic acid formed only acetic acid. The amount of butyric acid obtained from the mixture, corresponded with the amount of butyric acid formed from glucose alone.

VI. *2-Carbon Compounds*. The two substrates examined, glycolaldehyde and glycol, were not attacked by the bacteria within 48 hours. Glycolaldehyde also poisons the fermentation of glucose.

VII. *1-Carbon Compounds*. Formic acid (sodium formate in the presence of phosphate buffer (pH 6.2)) evolved not a single gas bubble after 48 hours incubation. From the mixture, after deproteinization, formic acid could be recovered unchanged. Inoculation of 5% maize mash with the mixture, led to vigorous fermentation. This proves that the viability of the bacteria was not impaired.

Inspection of Table VII leads to certain qualitative conclusions which we propose to discuss here.

In view of the good fermentability of pentitols and hexitols, one may well conclude from the non-fermentability of perseitol that heptoses are not capable of being attacked by *Cl. acetobutylicum*.

In the hexose series, aldoses and ketoses show no difference in fermentability, as far as the experiments go. The aldehyde group of the aldoses ( $C_1$ ) can be modified (reduced, oxidized, phosphorylated) without affecting qualitatively the fermentability, while the  $C_6$  hydroxyl must be present as such. Its phosphorylation greatly diminishes the amount of butyric acid formed, its oxidation to carboxyl (galacturonic acid) reduces it to zero. The behavior of saccharic acid (both  $C_1$  and  $C_6$  oxidized to carboxyl), although less extreme, points in the same direction; less butyric acid is formed.

It is noteworthy that quantitatively the well-fermentable  $C_6$ -compounds do not all behave alike. The sugars give a butyric/acetic ratio of almost 1.5:1, the hexitols 3-4:1, gluconic acid and hexose-1-phosphate 1:3-4. Whether these variations have any significance, can only be decided if in each case a complete analysis of the reaction products is available. However, Johnson, Peterson and Fred (17) have already suggested that the relative amounts of the various fermentation products depend on the substrate structure.

Most surprising and, it is believed, of more than qualitative importance, is the fact that hexose-1,6-diphosphate is converted into methylglyoxal and traces of pyruvic acid. This observation underlines the differences between the alcoholic and the acetone-butyl alcohol fermentation, as the Harden-Young ester is the point of attack in the alcoholic fermentation. It also shows that the typical  $C_3$  compounds are not the normal intermediates in the degradation of sugar to acetone and butyl alcohol (4a). The statement by Davies (18), that the diphosphate is not attacked, is incorrect. Davies was misled by the fact



TABLE VII  
*Classification of the Substrates Tested*

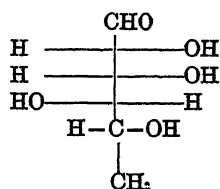
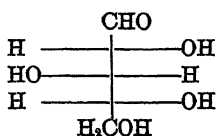
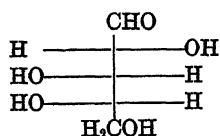
Group I. <i>Ratio butyric/acetic acid</i> > 1		
(a) < 2	Substrate:	Ratio butyric/acetic acid
	D-Glucose	1.6:1; 1:1
	L-Sorbose	1.5:1
(b) > 2	Substrate:	Ratio butyric/acetic acid
	D-Mannitol	4 : 1
	Dulcitol	4 : 1
	D-Sorbitol	3 : 1
	D-Arabitol	2.5:1
	Glycerol	3 : 1
Group II. <i>Ratio butyric/acetic acid</i> < 1		
	Substrate:	Ratio butyric/acetic acid
	D-Gluconic acid	1:3 1:5
	Saccharic acid	1:4
	L-Arabinose	1:2.5
	D-Xylose	1:2.5
	L-Rhamnose	1:3
	Hexose-1-monophosphate	1:2.5
	Hexose-6-monophosphate	1:10
Group III. <i>Substrates fermentable but not forming 4-carbon compounds</i>		
	Substrate:	Product formed
	D-Galacturonic acid	acetic acid
	Pyruvic acid	acetic acid
	Hexose-1,6-diphosphate	methylglyoxal
Group IV. <i>Substrates not attacked by the bacteria</i>		
	Perseitol	
	Monoacetoneglucose, Diacetoneglucose	
	Inositol	
	D-Arabinose	
	L-Arabinic acid	
	D-Arabinic acid	
	DL-Erythritol	
	DL-Glyceric aldehyde	
	3-Phosphoglyceric acid	
	Glycolaldehyde	
	Glycol	
	Formic acid	

that the attack is not accompanied by the gas evolution which is characteristic of the normal acetone-butyl alcohol fermentation.

From the observation that the grouping at the C<sub>1</sub> atom in hexoses is

not essential for fermentability, one could have foreseen that  $C_5$  compounds would behave like their  $C_6$  analogs. Indeed, it has been known for some time that the bacteria which are capable of metabolizing sugar to  $C_4$  compounds (butyric acid, butyl alcohol, butylene glycol) utilize pentoses as well as hexoses, and van der Lek (19) has even shown that not only are the same products formed but also that the yields are the same whether one ferments pentoses or hexoses. The only difference lies in the amount of carbon dioxide formed, and one is tempted to assume that the  $C_1$ -group in the  $C_5$  compounds is the main source of the carbon dioxide. Indeed, it is surprising that no attempt has been made by the authors who tried to formulate a theory of the acetone-butyl alcohol fermentation to account for the parallelism in behavior of hexoses and pentoses.

The experiments reported here do not as yet lead to any theory, either, but they demonstrate a few peculiarities of the  $C_5$  series of sugars. L-Arabinose, D-xylose and L-rhamnose are equally fermentable; D-arabinose is not attacked. D-Arabitol, on the other hand, is fermented.



Oxidation of the aldehyde groups at  $C_1$  destroys the fermentability of the pentoses, in contradistinction to the reduction to a primary hydroxyl. As to the butyric/acetic acid ratio, all  $C_5$  compounds rank with gluconic acid and hexose-1-phosphate rather than with the hexoses and the hexitols, but again, whether the difference is of any significance remains to be seen when the total fermentation products will have been determined in each case.

In view of the completely parallel behavior of D-xylose and the other two pentoses, the hypothesis of Guymon (20) that only *cis*-sugars are fermentable must be considered inadequate. In D-xylose the carbon atoms 2,3 and 4 have the *trans*, *trans*-configuration.

In view of the well-established fact that pyruvic acid is an obligatory intermediate in the carbohydrate metabolism of many types of cells, both in the animal and plant kingdoms, it has been very surprising

that the butanol-acetone producing organisms do not form  $C_4$ -compounds from that acid—neither fresh nor acetone-dried bacteria at any pH, in absence or presence of glucose—but give only acetic acid.

The pyruvic acid aldol hypothesis of Neuberg and Arinstein (21) refers to acetaldo, a 6-carbon compound. With pyruvic acid itself, these authors also obtained formic and acetic acid only. Besides, *Bacillus butylicus* Fitz, a microorganism which, according to Joergensen, differs completely from *Cl. butylicum*, had been used. References in the literature show that Bernhauer (21a) and other authors obtained butyl compounds from pyruvic acid also.

The statement to the contrary, made by Davies and Stephenson (6) and by Davies (18), could not be confirmed, nor was it possible to reproduce the experiments of Peldan (22) that addition of calcium ions makes possible the formation of butyric acid from pyruvic acid. It is worthy of note that Koepsell and Johnson (7) too, in experiments with a cell-free water extract from frozen cells of *Cl. butylicum*, obtained only acetic acid from pyruvic acid. The fact, incidentally, that this extract acts only upon pyruvic acid but does not attack glucose, supports the view that the dissimilation of pyruvic acid has nothing to do with the formation of 4-carbon compounds in the fermentation.

In accordance with Davies (18), phosphoglyceric acid, the precursor of pyruvic acid in other fermentations, was found not to be fermented. A mixture of phosphoglyceric acid and glycerophosphate also gave negative response. These results, in conjunction with the findings of Stone and Werkman (23) on the inability of butyric organisms to produce phosphoglyceric acid, show conclusively that the usual 3-carbon compounds cannot be considered as precursors of 4-carbon compounds, i.e., the scheme of the alcoholic fermentation cannot be applied to the fermentative production of acetone and butyl alcohol. DL-Glyceric aldehyde is not attacked either by *Cl. acetobutylicum*.

The only 3-carbon compound which forms 4-carbon compounds is glycerol. This fact, observed previously by Buchner and Meisenheimer (24), is best explained by the assumption that first a  $C_6$  compound is formed, in analogy to dihydroxyacetone, which is fermented by yeast after condensation to a hexose. Hexosemonophosphate has been isolated in that case (Neuberg and Kobel (25); Iwasaki (26)).

From the observation that glycerol gives the same high butyric/acetic acid ratio as the pentitols and hexitols, one will further conclude that it is condensed to a hexitol rather than to a hexose. It may be re-

called in this connection that according to Yamasaki and Simomura (27) *aspergilli* convert glycerol to mannitol.

As glycolaldehyde is not attacked by *Cl. acetobutylicum*, and even prevents the latter from fermenting glucose, Kluver's (28) hypothesis, that pentoses are split into glycolic aldehyde and a 3-carbon compound, during their fermentation, cannot be upheld. However, according to the many relevant experiments with *Fusaria* made by Nord and co-workers (29), the fermentability of pentoses is by no means an isolated phenomenon. Equally, formic acid cannot be regarded as the precursor of the gases (hydrogen and carbon dioxide) evolved in the fermentation, as is currently assumed. In accordance with Davies (18), it has been observed that it is not attacked by the acetone-butyl alcohol bacteria.

#### SUMMARY

1. The experiments reported here do not permit the formulation of a mechanism of the acetone-butyl alcohol fermentation. They may help to clear the way to such a mechanism by establishing certain new facts and by disproving some current assumptions.

2. Using fresh and acetone-dried bacteria, the formation of butyric acid was taken as the criterion for the convertibility of substrates into  $C_4$  compounds by *Cl. acetobutylicum* (Weizmann).

3. The formation of 4-carbon compounds from 6-carbon substrates is independent of the grouping (aldehyde, hydroxyl, carboxyl, phosphorylated hydroxyl) at the  $C_1$ -atom of the molecule.

4. The hydroxyl group at  $C_6$  seems to be essential for the formation of 3-carbon compounds, the latter being largely or completely suppressed by alterations in this group. This is especially conspicuous for hexose-1,6-diphosphate which is split into methylglyoxal and some pyruvic acid.

5. The natural pentoses and D-arabitol are fermented as well as hexoses and the hexitols. D-Arabinose is not fermented.

6. DL-Erythritol is not attacked by the bacteria.

7. The intermediate 3-carbon compounds of the alcoholic fermentation do not form 4-carbon compounds with *Cl. acetobutylicum*. In particular, pyruvic acid is not the precursor of the 4-carbon compounds.

8. Glycerol is the only 3-carbon compound which forms butyric acid. The possible mechanism of this transformation has been discussed.

9. The butyric/acetic acid ratio from various substrates depends upon the structure of the substrate.

10. The fermentability of D-xylose shows that *cis*-configuration of the substrate is not a necessary prerequisite.

11. Glycolaldehyde is not attacked by the bacteria and even inhibits the fermentation of glucose. The fermentation of pentoses cannot proceed through this aldehyde and a C<sub>3</sub> compound, as assumed previously.

12. Formic acid is not decomposed by the bacteria, nor does it poison or weaken them. The acid cannot be the precursor of the gases liberated in the normal fermentation

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# Mechanism of the Malt $\alpha$ -Amylase Action<sup>1</sup>

Karl Myrbäck

*From the Institute of Organic Chemistry and Biochemistry,  
the University, Stockholm, Sweden*

Received February 24, 1947

The  $\beta$ -amylase of grain and malt yields, on its action on starch or glycogen, maltose and a high molecular limit dextrin. The enzyme attaches itself to free, non-reducing end-groups of normally formed chains, i.e., chains of glucose residues united by the 1,4- $\alpha$ -glucosidic linkage (maltose linkage) (1). If, at a certain point in the molecule the structure deviates from this simple pattern, the enzyme action stops at this point (2). The second amylase present in malt, the  $\alpha$ -amylase, however, is not only independent of free end-groups, but its action is successively hindered by the proximity of end-groups. The enzyme has the capacity of attacking and rupturing any maltose linkage in a chain molecule of the starch type, but the velocity is greater for linkages at a distance from end-groups (3). In this respect branching points or other "anomalies" act as end-groups. The rapid action of the  $\alpha$ -amylase on linkages far from end-groups is due to a higher affinity of the enzyme to long normal chains than to chains shorter than a certain small number of glucose units (about 8) (4). Nevertheless, short normal chains are also split by the enzyme, but the velocity is small compared with the initial velocity of the action on starch or amylose.

In this paper some experiments will be described on the action of  $\alpha$ -amylase on a dextrin fraction, containing chiefly hexasaccharides with maltose linkages only. The isolation of the dextrin fraction is described in an earlier paper (5). The absence of anomalies (branchings, 1,6-glucosidic linkages) is shown by the fact that the dextrin is split to the extent of 100% into fermentable sugar by  $\beta$ -amylase (6).

The dry substance (5.52 g.) was dissolved in water, a few drops of a suitable phosphate buffer and 2 ml. of  $\alpha$ -amylase solution were added and the volume brought to 50 ml. The mixture was kept at 20°C. under toluol. At various times 1 ml. samples

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<sup>1</sup> Dedicated to Professor Carl Neuberg on his 70th birthday.



were removed and freed from toluol. The amount of fermentable sugar was determined by fermentation with bakers' yeast. As previously reported (7), the Swedish baker's yeast ferments glucose much more rapidly than maltose, so that the determination of glucose and maltose separately is possible in the same fermentation test. But earlier investigations (8, 9) from this laboratory have shown that maltotriose is fermentable by ordinary yeasts at approximately the same rate as maltose. If, therefore, maltotriose is present, the fermentation tests will give (a) the amount of glucose and (b) the amount of maltose + maltotriose. It is necessary to conduct several parallel fermentation experiments with known amounts of glucose and maltose.

The evolution of carbon dioxide from the 1 ml. tests are recorded in Fig. 1, some parallel experiments in Fig. 2.

From the curves are calculated the following values of fermentable sugar in 1 ml. of solution:

after 17.5 hours	12 mg. glucose + 30 mg. maltose (+ maltotriose)
after 65 hours	16 mg. glucose + 57 mg. maltose (+ maltotriose)
after 220 hours	16 mg. glucose + 72 mg. maltose (+ maltotriose)
after 500 hours	17 mg. glucose + 77 mg. maltose (+ maltotriose)

The dextrin solution contained 90.5 mg. of substance/ml.; this amount of a hexasaccharide would yield 94.5 mg. of sugar, calculated as maltose. The fermentation experiments, therefore, show, that the  $\alpha$ -amylase has transformed the dextrin completely into fermentable sugar.

The fact that  $\alpha$ -amylase (and, naturally, also "malt-amylase" as a whole) yields not only maltose but also glucose (which is *not* formed by secondary action of maltase) was mentioned previously by certain authors (10), but seems to have been almost forgotten. Experiments from this laboratory with  $\alpha$ -amylase and malt-amylase free from maltase have proven the direct formation of glucose beyond any doubt. The above experiments show that glucose is not formed only from starch but also from short chain saccharides with maltose linkages ( $\alpha$ -dextrins). This means that the enzyme has the capacity of attacking at least one of the terminal linkages in normal chain saccharides ( $n > 3$ ).

After 500 hours incubation the enzyme was destroyed by boiling. Fermentation experiments (Curve 5, Fig. 1) showed the presence of 17 mg. glucose and 78 mg. maltose (+ maltotriose)/ml. Samples were hydrolyzed at 100°C. with *N* HCl for 3-5 hours. Glucose determinations (Willstätter and Schudel) gave 102 mg. glucose/ml.; calculated 99 mg.

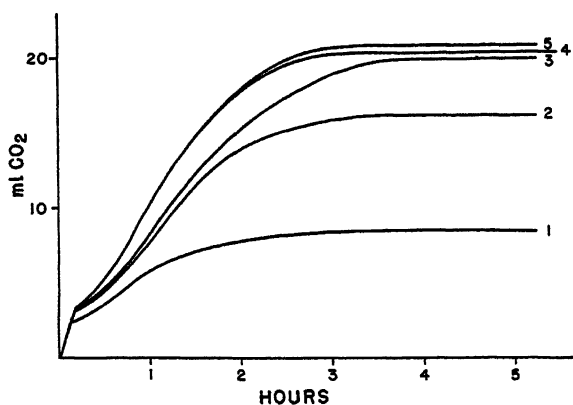


FIG. 1. Curve 1, after 17.5 hrs. incubation; Curve 2, after 65 hrs.; Curve 3, after 220 hrs.; Curve 4, after 500 hrs.; Curve 5, after boiling.

Before hydrolysis the reduction corresponded to an amount of 53.4 mg. glucose/ml. In reality only 17 mg. glucose/ml. were present, and a reduction value, corresponding to 37.4 mg. glucose, must be caused by other fermentable sugars. This reduction value corresponds to 71 mg. maltose, whereas the fermentation experiments gave a value of 78 mg. maltose, whereas the fermentation experiments gave a value of 78 mg. maltose. Then it must be concluded that maltotriose is also present, and a simple calculation gives the value 22 mg. for maltotriose. If all saccharides are

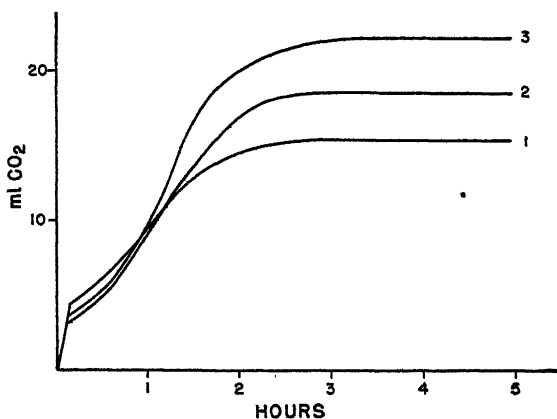


FIG. 2. Curve 1, 36.5 mg. glucose + 45.5 mg. maltose; Curve 2, 10.0 mg. glucose + 74.0 mg. maltose; Curve 3, 16.0 mg. glucose + 92.0 mg. maltose.

calculated as glucose we find that the action of malt  $\alpha$ -amylase on the hexasaccharide has resulted in a mixture of 17% glucose, 60% maltose and 23% maltotriose.

The enzyme does not attack maltose or maltotriose. The glucose must be derived from saccharides with 4-6 glucose units. If, for instance, one glucose molecule is split off from a hexasaccharide, a pentasaccharide results, which on further action of the enzyme may yield one molecule each of maltose and maltotriose or perhaps one molecule of glucose and two molecules of maltose. But formation of maltotriose may also occur by scission of the central linkage in the hexasaccharide, and finally the formation of three molecules of maltose from the hexasaccharide also seems possible. Probably all these reactions proceed simultaneously with different velocities. The experiments are in good agreement with the assumption that the enzyme can attack substantially all normal glucosidic linkages in a chain molecule with velocities varying with the distance of the linkage in question from the end-groups. As reported before, the experiments show that maltotriose is fermentable but is not attacked by  $\alpha$ -amylase.

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# On the Fermentation of Carbon Monoxide by Pure Cultures of Methane Bacteria<sup>1</sup>

A. J. Kluyver and Ch. G. T. P. Schnellen

*From the Laboratory for Microbiology, Technische Hoogeschool, Delft, Holland*

Received February 19, 1947

## INTRODUCTION

Fermentation is often defined as the splitting of a substrate into two or more fragments, part of which are oxidized and part reduced as compared with the constitution of the original compound acting as the substrate. Seen in this light the simple molecule of carbon monoxide does not seem to offer any prospect for its applicability as a fermentation substrate. If, however, a somewhat broader definition of fermentation, *viz.*, an anaerobic conversion of a compound into more oxidized and into more reduced molecules, is considered, the case for carbon monoxide appears in a new light. For then it is clear that the intermediary position of this compound between carbon dioxide as ultimately oxidized carbon and methane as ultimately reduced carbon implies a potentiality which might well be successfully exploited in microbial life.

Some 15 years ago investigations carried out by Fischer, Lieske and Winzer (1) have definitely shown that nature has not neglected this chance, inasmuch as experimental proof has been given that sludge derived from an anaerobic sewage fermentation tank ("Emscherbrunnen") is able to bring about an anaerobic conversion of carbon monoxide into carbon dioxide and methane.

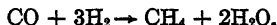
The starting point of the investigations of Fischer *et al.* is to be found in Söhngen's classical observation that highly purified cultures of methane bacteria are able to convert a mixture of carbon dioxide and hydrogen into methane (2). The German investigators, in their endeavor to apply this biological reaction technically, extended their observations to the behavior of a crude culture of methane bacteria toward a mixture of carbon monoxide and hydrogen. It was then found that this mixture was also converted into methane. A study of the influence of variations in the composition

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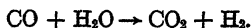
<sup>1</sup> Dedicated to the "Altmeister" of fermentation study, Professor Carl Neuberg, on the occasion of his 70th anniversary.

of this mixture led almost automatically to an investigation of the action of the said culture on pure carbon monoxide with the result already mentioned above.

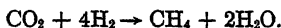
Although by far the greater part of the experiments carried out by Fischer *et al.* dealt with the conversions in complex industrial gas samples, such as illuminating gas, the authors arrived at the conclusion that the consumption of CO in these samples was not due, as might be expected, to the simple reaction:



They claim they have found indications in favor of the hypothesis that the primary reaction in which the carbon monoxide takes part is always:



In the presence of a sufficient amount of hydrogen the primary products disappear again completely because of the secondary reaction:



They also extend this view to the interpretation of the only experiment in which pure carbon monoxide has been used.

While the conversion into carbon dioxide and methane is fully borne out by experimental evidence, the main arguments for the proposed intermediary metabolism in this case are to be found in a slight increase of gas pressure during the first phase of the experiment—asccribed to the first reaction—and in the fact that at the end of the experiment (after 54 days) a small quantity of hydrogen (1%) appeared to be present in the gas mixture.

In addition to these observations it was found that, in the initial stages of the experiments on the gas samples containing carbon monoxide and hydrogen, as a rule part of the carbon monoxide disappeared without equivalent production of methane or of methane and carbon dioxide. This could only mean that reactions between carbon dioxide, carbon monoxide and hydrogen had occurred, leading to some non-gaseous products which, accordingly, must have accumulated in the medium. In the later stages of the fermentation this product was apparently again decomposed into methane and carbon dioxide. This intermediary product could then be identified as acetic acid.

A continued investigation of this unexpected observation led to a second publication by the same authors (3). In this article the production of acetic acid by crude cultures in contact with a mixture of carbon dioxide (or carbon monoxide) and hydrogen is extensively studied and it is concluded that this acid is a regularly occurring intermediary product in the conversion of such a gas mixture into methane.

In 1933 Stephenson and Stickland (4) published their investigations on what they claim to be a one-cell culture of a formate-fermenting methane bacterium. They ascribe to this organism the ability to convert not only carbon dioxide and carbon monoxide, but also formic acid, formaldehyde (as hexamethylenetetramine), and methyl alcohol into methane by means of gaseous hydrogen. However, the organism was unable to reduce organic compounds with more than one carbon atom.

Since then our general knowledge of methane fermentation has been considerably extended. In a number of publications Barker (5, 6, 7) has produced experimental proof that, also in the production of methane from organic substrates, in all cases a

reduction of carbon dioxide is involved so that methane fermentation can be represented by the general equation:

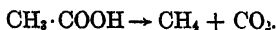


Moreover, Barker (8) was the first investigator to succeed in developing a method for obtaining pure cultures of methane-producing bacteria.

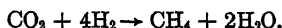
Another significant contribution was, however, made by Wieringa (9) who carried on a more detailed study of the conversion of a mixture of carbon dioxide and hydrogen to methane. Wieringa, too, observed a production of acetic acid when a crude culture acted on this gas mixture, but he could bring experimental proof that this conversion was caused by a spore-forming, non-methane-producing bacterium to which the name *Clostridium acetium* was given. In the enriched cultures studied by him methane production was apparently due to two consecutive reactions. The first of these caused by *Cl. acetium* proceeded according to the equation:



This reaction was then followed by a second reaction brought about by some true methane bacterium:



It will be clear that these findings again cast considerable doubt on the ability of pure cultures of methane bacteria to bring about a direct conversion of carbon dioxide and hydrogen according to the equation:



However, in an extensive study on methane fermentation one of the present authors (10) could prove experimentally that pure cultures of three different species of methane bacteria are indeed able to reduce carbon dioxide with gaseous hydrogen to methane, the same result having also been reported meanwhile by Barker (11) for his *Methanobacterium Omelianskii*.

Under these conditions it seemed of importance to reinvestigate also the problem of biological methane production from carbon monoxide, and to study more especially in how far pure cultures of methane bacteria are able both to reduce this compound with gaseous hydrogen and to bring about a carbon monoxide fermentation in the absence of hydrogen. For it seems scarcely open to doubt that in the experiments of Fischer *et al.* the crude cultures used also contained Wieringa's *Cl. acetium*, and that the observed intermediary formation of acetic acid has been due to the presence of this organism. Moreover, the fact that the German authors had been working with a mixture of bacteria also left open the possibility that the primary conversion of carbon monoxide into carbon dioxide and hydrogen does not occur under the influence of the methane bacteria, but is brought about by some synergistic organism like *Cl. acetium*.

## EXPERIMENTAL

## 1. The Organisms Used and Their Cultivation

Enrichment cultures in which methyl alcohol and calcium formate, respectively, acted as substrate led to the isolation of pure cultures of two different species of methane-producing bacteria. For all details regarding this isolation, carried out chiefly according to the technique of Barker (8), we refer to Schnellen (10). The methyl alcohol-fermenting species, for which the name *Methanosarcina Barkerii* has been proposed, proved capable of fermenting acetates as well, although with difficulty. None of the many other organic salts tested, nor any of the alcohols other than methyl alcohol, were fermented. As for the formate-fermenting *Methanobacterium formicicum* we have been unable to find any other fermentable substrate beside the formate.<sup>2</sup>

The third pure culture we had at our disposal was *Methanobacterium Omelianskii*. We obtained this culture from Dr. H. A. Barker of the University of California at Berkeley, who, in 1939, had kindly sent us a transfer of his isolation.

All three species were cultivated in anaerobic culture tubes according to Hall as modified by Barker (8). The medium used for the cultivation of *Mb. Omelianskii* was the same as that originally used by Barker. To 100 ml. of tapwater 1%  $K_2HPO_4$ , 0.05%  $NH_4Cl$  and 0.05%  $MgSO_4$  were added. After sterilization, 8 ml. of a 10% solution of  $Na_2CO_3$ , 1 ml. of a 1% solution of  $Na_2S$  and 1 ml. of ethyl alcohol (96%) were added, and the pH adjusted to 7.0 with 8% HCl.

For the cultivation of *Ms. Barkerii* the same medium was used, except that the ethyl alcohol was replaced by 1.5 ml. methyl alcohol.

For *Mb. formicicum* the medium was prepared as follows. To 100 ml. of tapwater 1%  $K_2HPO_4$ , 0.05%  $(NH_4)_2SO_4$  and a trace of  $FeSO_4$  were added. After sterilization, 3.5 ml. of a sterile solution of sodium formate (20%) and 3 ml. of a 1% solution of  $Na_2S$  were added and the pH again adjusted to 7.0.

In Hall tubes containing 35 ml. of the medium, fermentation was usually completed after 8-14 days. After this period, in each tube about 2 ml. of a more or less flocculate sediment was present; for each experiment on gas conversion 10 ml. of sediment containing the greater part of the bacteria present in five tubes were ordinarily used.

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<sup>2</sup> It should be remarked that this bacterium was not identical with the organism used by Stephenson and Stickland, as mentioned above, since our culture did not produce any methane from hexamethylenetetramine or methyl alcohol in a hydrogen atmosphere.

## 2. Apparatus Used for the Study of the Gas Conversion and Analytical Procedure

The apparatus used for the study of the gas conversion was simple and can best be characterized as a developed model of the fermentation tube of Eldredge (12). The only difference was that one of the vertical tubes was closed with a well fitting glass stopper, while the other was connected by a ground glass joint with a narrow glass tube provided with a glass stopcock.

The volume of the apparatus was accurately determined by weighing before and after filling with mercury.

Before the experiment the glass joint and the stopcock were replaced by cotton plugs. Then the fermentation vessel, together with the two separate parts, were sterilized by dry heat.

For each experiment exactly 10 ml. of the bacterial suspension was placed in one of the horizontal arms, whereupon the corresponding vertical tube was closed with the glass stopper. Then the second cotton plug was replaced by the glass joint with the stopcock and now the apparatus as a whole was evacuated.

Next the apparatus was filled with a known volume of the gas mixture previously prepared in a gas burette.

The apparatus was then placed in an incubation room at 30°C. where it was subjected to continuous shaking to ensure close contact between the bacteria and the gas phase.

At the end of the experiment the gas in the vessel was brought back to atmospheric pressure by adding a measured quantity of hydrogen from a burette. Hereupon, an analysis of the resulting gas phase was made, and, by subtracting the hydrogen added, the original composition of the gas phase could be calculated.

The analysis of the residual gas mixture, in which the possible presence of hydrogen, carbon monoxide, carbon dioxide and methane had to be taken into account, was performed according to the usual gasometric procedure.

## 3. The Conversion of a Mixture of Carbon Monoxide and Hydrogen by *Methanosarcina Barkeri*

A series of experiments was started in which the percentage of carbon monoxide in the mixture varied from about 10 to 25%. Analysis was made after five days.

The results of the experiments are shown in Table I.

In all cases the carbon monoxide had been completely consumed at the end of the experiment.

A recalculation of the experimental data leads to the figures presented in Table II.

It needs no comment that, within the limits of experimental error, these data are in excellent agreement with the assumption that *Meth-*

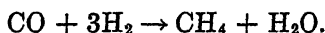


TABLE I

*Conversion of Carbon Monoxide-Hydrogen Mixtures by Methanosarcina Barkerii*

Exp. No.	Initial gas volumes ml. 760 mm./0°C.		Carbon monoxide concentration per cent	Residual gas volumes ml. 760 mm./0°C.		
	H <sub>2</sub>	CO		H <sub>2</sub>	CO	CH <sub>4</sub>
1	70.0	8.3	10.6	47.9	—	7.6
2	70.6	8.7	11.0	48.1	—	7.8
3	70.3	11.8	14.4	39.7	—	10.3
4	64.4	12.7	16.5	28.2	—	12.6
5	60.1	19.2	24.2	10.0	—	17.3

*anosarcina Barkerii* in pure culture is able to bring about the conversion:



To get a closer idea of the velocity of the conversion, it was decided to start another series of experiments in which the duration was limited to three days.

TABLE II

*Comparison of Actual and Theoretical Quantities of Methane Formed in the Conversion of Carbon Monoxide-Hydrogen Mixtures by Methanosarcina Barkerii*

Exp. No.	Hydrogen consumed mM	Carbon monoxide consumed mM	Methane produced mM	Methane produced calculated on hydrogen consumed (1/3)
1	1.00	0.36	0.34	0.33
2	1.01	0.39	0.35	0.34
3	1.39	0.53	0.46	0.46
4	1.63	0.57	0.56	0.55
5	2.26	0.86	0.77	0.76

The results obtained in this series are given in Table III.

As is recorded in this table, the conversion is still quite incomplete in three days. We observed, however, in these experiments that this time carbon dioxide was also present unexpectedly among the products of the conversion. Although, in the first three experiments, the quantities determined are but small, they are still significant considering that

TABLE III  
*Incomplete Conversion of Carbon Monoxide-Hydrogen Mixtures  
 by Methanosarcina Barkerii*

Exp. No.	Initial gas volumes ml. 760 mm./0°C.		Carbon monoxide concentration per cent	Residual gas volumes ml. 760 mm./0°C.			
	H <sub>2</sub>	CO		H <sub>2</sub>	CO	CO <sub>2</sub>	CH <sub>4</sub>
6	65.5	7.4	10.1	57.3	4.2	1.0	2.5
7	64.9	12.7	16.4	60.1	10.3	0.8	1.5
8	56.9	18.0	24.1	48.5	14.0	1.1	2.5
9	51.2	16.9	24.8	48.2	5.1	5.8	2.5

in these analyses the amount of carbon dioxide dissolved in the medium remained unaccounted for.

After what has been said in the Introduction regarding the theory presented by Fischer *et al.* on the primary conversion of carbon monoxide, it is clear that the demonstration of carbon dioxide as an intermediary product strongly favors the idea that the reduction of carbon monoxide by *Ms. Barkerii* indeed proceeds by two steps as suggested by the said authors:



As long as there is at least a three-fold surplus of hydrogen in the initial gas atmosphere this special reaction course remains undetected when the conversion has reached completion.

#### 4. The Conversion of Carbon Monoxide by *Ms. Barkerii* without Addition of Hydrogen

The occurrence of carbon dioxide as an intermediate product in the foregoing experiments strongly suggests that *Ms. Barkerii* will also be able to attack carbon monoxide in the absence of hydrogen, and it was, therefore, decided to test this possibility experimentally.

Since it seemed possible that *Ms. Barkerii* would only be able to tolerate relatively low pressures of carbon monoxide it appeared advisable to supply the bacteria in the following experiments with mixtures of carbon monoxide and nitrogen in which the concentration

of the carbon monoxide varied from about 20 to 100%. The experiments were again extended over five days.

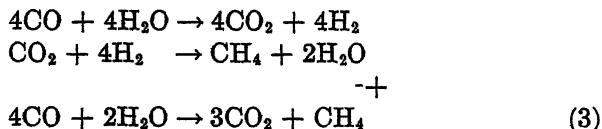
The experimental data obtained are given in Table IV.

TABLE IV  
*Conversion of Varying Quantities of Carbon Monoxide by  
Methanosarcina Barkerii*

Exp. No.	Initial volume of carbon monoxide ml. 760 mm./0°C.	Carbon monoxide concentration per cent	Residual gas volumes ml. 760 mm./0°C.		
			CO	CO <sub>2</sub>	CH <sub>4</sub>
10	15.6	19.9	—	9.3	4.2
11	18.3	25.0	—	10.5	4.8
12	25.5	32.5	3.2	12.0	5.6
13	39.1	49.3	20.1	9.3	5.0
14	37.8	49.4	18.6	13.0	5.3
15	76.6	100.0	66.8	5.0	2.4

According to the data recorded in Table IV large quantities of carbon dioxide have been formed in all cases, a result which supports the concept that the first conversion of carbon monoxide proceeds according to reaction (1). Evidently this primary conversion is followed by reaction (2) with the restriction, however, that the hydrogen present will only suffice to reduce one-quarter of the amount of carbon dioxide produced.

This means that the fermentation of carbon monoxide as a whole will proceed according to the equations:



In Table V the experimental quantities of methane produced have been compared with the theoretical quantities calculated on the basis of equation (3).

It is evident that there is good agreement between these values. The fact that part of the carbon dioxide has remained dissolved in the medium explains that the experimental figures for this gas remain somewhat below the theoretical values.

TABLE V

*Comparison of Actual and Theoretical Quantities of Methane Formed  
in the Fermentation of Carbon Monoxide by  
Methanosarcina Barkerii*

Exp. No.	Initial quantity of carbon monoxide mM	Carbon monoxide consumed mM	Carbon monoxide consumed per cent of the initial quantity	Methane produced mM	Methane produced calculated on carbon monoxide consumed (1/4)
10	0.70	0.70	100	0.19	0.18
11	0.82	0.82	100	0.22	0.21
12	1.14	1.00	88	0.25	0.25
13	1.75	0.85	49	0.22	0.21
14	1.70	0.86	51	0.24	0.22
15	3.42	0.47	14	0.11	0.12

A most remarkable result is undoubtedly that, even in an atmosphere of 100% carbon monoxide, *Ms. Barkerii* brings about a quite significant conversion of this gas, so extremely poisonous for by far the majority of all living beings.

Although the results obtained in the last series of experiments are in themselves a support for the correctness of the proposed reaction course, it was tempting to try to bring direct proof for the occurrence of hydrogen as an intermediate product. It was considered that a fermentation of carbon monoxide in the presence of strong alkali which would tend to absorb all carbon dioxide from the gas phase might lead to an accumulation of hydrogen.

In an experiment performed under these conditions the 15.6 ml. of carbon monoxide initially present in the gas phase had been consumed completely after 5 days. An analysis of the residual gas showed the presence of 2.6 ml. of methane and 4.6 ml. of hydrogen.

It is evident that, if all the carbon dioxide had been trapped by the alkali, 15.6 ml. of hydrogen should have been found in the final analysis. This means that 15.6 - 4.6 = 11.0 ml. hydrogen have still been consumed for reduction of the carbon dioxide formed. Accordingly 2.7 ml. of methane should have been produced, a result which is in agreement with the experimental value of 2.6 ml. It should be realized that, of the 15.6 ml. of carbon dioxide primarily formed, only 2.6 ml. have been converted into methane, while 13.0 ml., or 83% of the total amount, have been absorbed by the alkali.

Altogether, it may be concluded that this experiment has brought irrefutable proof for the fact that the primary conversion of carbon

monoxide by *Ms. Barkerii* is indeed the production of carbon dioxide and hydrogen.

5. *The Conversion of a Mixture of Carbon Monoxide and Hydrogen by Methanobacterium formicicum*

Similar experiments to those described in §3 for *Ms. Barkerii* were also carried out with *Mb. formicicum*.

It soon became evident that with the latter organism also a reduction of the carbon monoxide was brought about, but that the conversion of the gas mixture only went to completion if the carbon monoxide concentration was kept low.

The results reported in Table VI demonstrate this clearly. With a carbon monoxide concentration of 12.3% this gas is again completely consumed; however, at a concentration of 14.2% the conversion is still incomplete after 5 days.

TABLE VI  
*Conversion of Carbon Monoxide-Hydrogen Mixtures  
by Methanobacterium formicicum*

Exp. No.	Initial gas volumes ml. 760 mm./0°C.		Carbon monoxide concentration <i>per cent</i>	Residual gas volumes ml. 760 mm./0°C.		
	H <sub>2</sub>	CO		H <sub>2</sub>	CO	CH <sub>4</sub>
16	72.6	8.66	10.6	46.9	—	9.0
17	67.3	9.4	12.3	37.7	—	9.9
18	68.3	11.5	14.5	43.2	4.2	7.9
19	67.6	11.2	14.2	47.0	5.1	6.7

A recalculation of these data is presented in Table VII.

From these data it is evident that the amount of methane formed agrees in a very satisfactory way with the amount calculated on the basis of hydrogen consumption.

Also with *Mb. formicicum* an experiment was made in a gas phase consisting of carbon monoxide and nitrogen. Although the results in this case were somewhat impaired by the low carbon monoxide concentration which could be applied (11.6%) a production of carbon dioxide and methane in amounts approximating the theoretical values could be established with certainty.

TABLE VII

*Comparison of Actual and Theoretical Quantities of Methane Formed  
in the Conversion of Carbon Monoxide-Hydrogen  
Mixtures by Methanobacterium formicicum*

Exp. No.	Hydrogen consumed mM	Carbon monoxide consumed mM	Methane produced mM	Methane produced calculated on hydrogen consumed (1/3)
16	1.16	0.39	0.40	0.39
17	1.32	0.42	0.44	0.44
18	1.12	0.33	0.36	0.37
19	0.93	0.27	0.30	0.31

### 6. Experiments with *Methanobacterium Omelianskii*

Finally a number of experiments were carried out with *Mb. Omelianskii*. Here, however, no conversion of a carbon monoxide-hydrogen mixture was ever observed.

Since it seemed possible that this negative result was either due to a lack of activity of the cultures applied, or was caused by a great sensitivity of this bacterium toward higher concentrations of carbon monoxide, it seemed of importance to investigate the behavior of the bacterium toward a gas mixture in which, besides hydrogen, both carbon monoxide and carbon dioxide were present.

The results of three experiments are recorded in Table VIII.

TABLE VIII

*Conversion of Gas Mixtures Consisting of Carbon Monoxide, Carbon  
Dioxide and Hydrogen by Methanobacterium Omelianskii*

Exp.	Initial quantities mM			Residual quantities mM			Methane pro- duced calculated on hydrogen consumed (1/4)
	CO	CO <sub>2</sub>	H <sub>2</sub>	CO	H <sub>2</sub>	CH <sub>4</sub>	
20	0.31	0.31	2.66	0.31	1.40	0.31	0.32
21	0.34	0.34	2.62	0.34	1.32	0.31	0.32
22	0.36	0.36	2.82	0.36	1.36	0.36	0.36

The figures in Table VIII show conclusively that the carbon monoxide has remained untouched, while all the carbon dioxide has been reduced. The quantity of methane found is in perfect agreement with

the quantity calculated on the assumption that all hydrogen consumed has been used for the reduction of the carbon dioxide.

The inability of *Mb. Omelianskii* to reduce carbon monoxide offers a new argument that this compound is not an intermediate product in the reduction of carbon dioxide in methane fermentation, and, therefore, indirectly supports the idea that those methane bacteria which attack carbon monoxide convert this compound primarily into carbon dioxide and hydrogen.

### DISCUSSION

After Barker's work had opened the possibility of obtaining various methane bacteria as pure cultures, it became feasible to submit the question of whether methane bacteria alone would be able to reduce carbon dioxide to methane to a direct test. Schnellen (10), and also Barker (11), have since shown that several species of methane bacteria indeed bring about a direct reduction of carbon dioxide with gaseous hydrogen, and that, accordingly, the occurrence of acetic acid in the experiments of Fischer *et al.* and of Wieringa, must have been due to the incidental simultaneous presence of *Cl. aceticum* in the media.

Under these conditions it seemed of importance to study also the behavior of pure cultures of methane bacteria toward carbon monoxide.

The investigation of Stephenson and Stickland (4) suggested that it might be advantageous to use for this study cultures of organisms which had been found able to ferment other compounds with one carbon atom. For this reason pure cultures of two new species of methane bacteria, viz., *Methanosarcina Barkerii* and *Methanobacterium formicum*, respectively isolated from a methane fermentation of methyl alcohol and of calcium formate, were used in this investigation. Moreover, a pure culture of the methyl alcohol-fermenting *Methanobacterium Omelianskii*, kindly put at our disposal by Dr. H. A. Barker, was included in the investigation.

Our experiments with the two first mentioned species have shown conclusively that both methane bacteria are able to convert a mixture of carbon monoxide and hydrogen into methane.

In experiments with *Ms. Barkerii*, carbon dioxide was found to be present when the gas mixture was analyzed before the conversion of the carbon monoxide had reached completion. This result is strongly in favor of the view of Fischer *et al.* that the primary reaction into which carbon monoxide enters is the reaction with water to give carbon dioxide

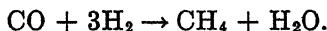
and hydrogen and, moreover, proves that this reaction is a metabolic process of the methane bacterium itself. Further proof of this was obtained in experiments in which *Ms. Barkerii* acted on carbon monoxide in the absence of hydrogen. Here the carbon monoxide was quantitatively converted into carbon dioxide and methane, thus clearly demonstrating the ability of the organism to produce carbon dioxide from carbon monoxide. In a special experiment, in which, during the conversion, the carbon dioxide was removed from the gas phase by strong alkali, it was possible also to give experimental proof for the formation of hydrogen.

The experiments with *Mb. formicicum* led in principle to the same results as those with *Ms. Barkerii*. However, to attain a complete conversion of the carbon monoxide in presence of an excess of hydrogen, it proved necessary to keep the concentration of carbon monoxide relatively low ( $\pm 12\%$ ).

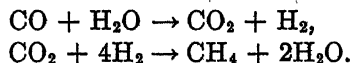
In contrast to the two foregoing species of methane bacteria *Mb. Omelianskii* proved to be completely unable to attack carbon monoxide. In experiments in which a mixture of carbon dioxide, carbon monoxide and hydrogen was tested, the carbon dioxide was converted into methane, while the carbon monoxide was quantitatively recovered. This negative result is interesting because it gives independent proof that, in the reduction of carbon dioxide by methane bacteria, carbon monoxide is not an intermediate product. To the contrary, it has now been proved that the reverse situation holds.

#### SUMMARY

The anaerobic conversion of carbon monoxide under the influence of pure cultures of methane bacteria has been studied. It has been found that the new species *Methanosarcina Barkerii* which has been isolated from a methane-producing enrichment culture with methyl alcohol as sole organic compound is able to convert a mixture of carbon monoxide and hydrogen according to the ultimate equation:



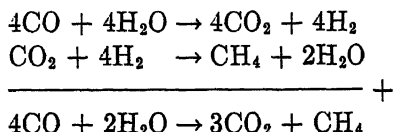
Evidence has, however, been presented that this fermentation process actually proceeds in two steps:





In agreement herewith it has been established that *Ms. Barkerii* also acts on carbon monoxide in the absence of hydrogen.

In this case the reaction course can be represented as follows:



*Ms. Barkerii* can bring about this conversion even in an atmosphere of 100% CO.

For the new formate-fermenting *Methanobacterium formicicum*, in principle, the same situation holds as for *Ms. Barkerii*, with the distinction, however, that a complete conversion of a carbon monoxide and hydrogen mixture can only be attained with low concentrations of carbon monoxide.

In contrast to the two foregoing species *Methanobacterium Omelianskii* is completely unable to attack carbon monoxide. In a mixture of carbon dioxide, carbon monoxide and hydrogen the first compound is reduced to methane, while the carbon monoxide remains unaffected.

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# The Rate of Turnover of Hexosediphosphate in Brain Preparations<sup>1,2</sup>

Otto Meyerhof and Jean R. Wilson

*From the Department of Physiological Chemistry, School of Medicine,  
University of Pennsylvania, Philadelphia*

Received February 18, 1942

## 1. INTRODUCTION

That brain extracts glycolyze by way of phosphorylated compounds and metabolize hexosediphosphate (HDP) rapidly, was shown some years ago (1). Creatine present in brain could serve as P-acceptor. These findings were later extended by the author (2), as well as by others (3, 4, 5), and every step of the phosphorylating glycolysis was shown to be going on at high speed in tissue and extract from brain as well as tumor, embryo, retina, kidney, liver, *etc.*

While, therefore, no doubt exists as to the pathway of anaerobic sugar breakdown in mammalian tissues, the factors which control the rate of turnover are still known only partly. Recently it was shown by us (6, 6a) that the rate of glycolysis of the dissolved enzymes of brain greatly exceeds the actual rate encountered in the living tissue.  $Q_{L_a}$  values ( $\text{mm}^3 \text{ CO}_2$  liberated by lactic acid/mg. dry weight/hr. at  $38^\circ\text{C}.$ ) of 50–70 with glucose or fructose are obtained in extracts, while tissue slices and homogenates give  $Q_{L_a}$  values of 7–15 for glucose and 3–7 for fructose.

The affinities of the two enzymes hexokinase and adenylpyrophosphatase ("apyrase")<sup>3</sup> are mainly responsible for these differences.

<sup>1</sup> Dedicated to Professor Carl Neuberg on his 70th birthday.

<sup>2</sup> This work was aided by grants from the Baird Foundation and the Daxian Foundation.

<sup>3</sup> Abbreviations used: ATP = adenosinetriphosphate; ADP = adenosinediphosphate; AA = adenosine-5-monophosphate; ATPase = enzyme splitting first labile P-group of ATP; apyrase = adenylpyrophosphatase, enzyme splitting both labile P groups; Pyro-P = labile P of ATP and ADP; HDP = hexosediphosphate; CP = creatinephosphate.

It was shown that the high concentration of apyrase in the brain homogenate lowers the concentration of ATP below the optimal level. In the centrifuged extract, on the other hand, the apyrase is mostly removed and the sugar turnover is accordingly high, because of the high ATP-concentration.

The problem is studied here more in detail with the help of the turnover rate of HDP. Aside from the function of P acceptors like creatine, another way of bringing about a high turnover rate of HDP consists in having a highly active apyrase. As was shown recently, the low fermentation of HDP in yeast extracts could be raised above the level of fermentation of glucose by addition of purified apyrase from potatoes (7). Since all other partial enzymes of fermentation are present in excess, the amount of apyrase controls the speed. As will be shown, a similar situation exists in the extract of brain, but not in the homogenate, where the apyrase is in excess compared to the other glycolytic enzymes.

## 2. METHODS AND PREPARATIONS

Methods and preparations were mostly the same as in the foregoing paper (6). Hexosediphosphate was purified from the commercial calcium salt (Schwarz Laboratories, New York) by removing the calcium and reprecipitating with barium acetate at pH 3.1 with gradual addition of one volume of ethyl alcohol. In this way hexosemonophosphate is removed. For hexosemonophosphate we used Robison ester prepared from dry yeast as Ba salt (P content 6.4%). ATP and cozymase were the same preparations used formerly (6). CP was prepared according to Meyerhof, Schulz and Schuster (8).<sup>4</sup> Homogenates were made 5:1 with 3 parts of modified Ringer solution (containing Mg instead of Ca) and two parts of isotonic  $\text{NaHCO}_3$  as described formerly.

## 3. DISTRIBUTION OF APYRASE IN BRAIN TISSUE

Dubois and Potter (9) found a very active apyrase (or ATP-ase) in all mammalian tissues, somewhat less in brain than in other organs, 1 mg. of fresh brain tissue splitting off 2.4  $\gamma$  of pyro-P in the absence of  $\text{Ca}^{++}$  and 7  $\gamma$  in the presence of optimal  $\text{Ca}^{++}$  in 15 min. at 38°C. Our values are not strictly comparable, because the homogenate was made in Ringer solution (without Ca), and Mg ( $5 \times 10^{-4}$  M) was present instead. Calculated for 15 min. the turnover would amount to 14  $\gamma$  pyro-P split for 1 mg. of fresh brain. Some of the results are shown in Fig. 1. Incubation times were 1, 2 and 3 min. for the homogenate and

<sup>4</sup> We thank Dr. Wayne Kielley for this preparation.

2 and 10 min. for the centrifuged extract. With 0.3 cc. homogenate (1:5) in 0.8 cc. total volume and 90–100  $\gamma$  pyro-P, the speed drops even after one minute, because of the complete splitting off of the first P group. With 0.15 cc. homogenate and the same amount of pyro-P the rate remains constant for two min.

Nine-tenths of this activity is connected with the structural elements. After removing them by centrifugation, the opalescent, but clear ex-

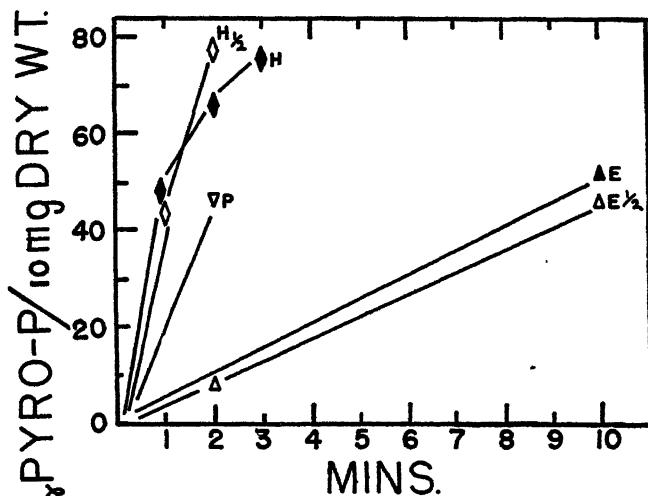


FIG. 1. Activity of Apyrase in Homogenate (H), Centrifuged Extract (E), and Washed Particles (P). 0.3 cc. or 0.15 cc. Ringer-homogenate or extract or 0.3 cc. particles washed in Ringer solution are used. 0.2 cc. ATP with 90  $\gamma$  pyro-P added in the beginning. Total vol. 0.8 cc. Ordinate pyro-P split off, determined as difference between 7-min. P and direct P. The dry weight refers to the amount of tissue taken, not to the amount in solution 38°C.

H  $\blacklozenge$  0.3 cc. homogenate; H½  $\diamond$  0.15 cc. homogenate.

E  $\blacktriangle$  0.3 cc. extract from this homogenate; E½  $\triangle$  0.15 cc. extract.

P  $\nabla$  0.3 cc. particles filled, after washing, to the original volume of the homogenate.

tract shows an activity which is one-tenth that of the complete homogenate, both when 0.3 cc. and when 0.15 cc. are taken (Fig. 1). If the structural elements are washed twice with Ringer solution and filled up to the original volume of the homogenate, they have still retained about half of the activity of the homogenate, in spite of further loss of protein by washing; they split off as much pyro-P in 2 min. as the extract does in 10 min.

The dephosphorylation of ATP is due to the apyrase, but a non-specific phosphatase is also present and is mainly bound to the structures. Its activity is about a tenth of that of the apyrase, as can be shown with splitting of glycerophosphate or of the third P group of ATP. For shorter times the influence of this phosphatase is negligible, but in manometric measurements of glycolysis over an hour, its role is important, because the AA is dephosphorylated irreversibly in this way. With the usual amount of ATP added (50  $\gamma$  pyro-P/cc., with 0.3 cc. homogenate 1:5) all 3 groups of ATP would be dephosphorylated in the absence of P acceptors in 15 min. In the presence of P acceptors this time is prolonged by partial regeneration of ATP. But, because the apyrase prevails overwhelmingly over the other enzymes of the glycolytic cycle, the irreversible loss of AA occurs rather rapidly and contributes to the rapid decay of the glycolysis of free sugars in the homogenate, unless fresh ATP is repeatedly added (*cf.* 6). In the centrifuged extract, on the other hand, very little of this non-specific phosphatase is left; it would take much more than an hour in this case to split off the third phosphate of the added ATP.

When the homogenate is made with distilled water, the apyrase apparently becomes more unstable. The high rate of sugar turnover in the water homogenate found by Utter *et al.* (5), which we obtained sometimes but not regularly under the same conditions, can be ascribed partly to inactivation of the apyrase. (See also section 5.) This is in line with the inactivation of myosin-ATPase and yeast apyrase by prolonged exposure to distilled water. It explains moreover the observation of Utter *et al.* that, after centrifugation at 18,000 r.p.m., their extract can glycolyze hexosediphosphate only in the presence of arsenate, an indication that the apyrase is completely removed. This does not happen with Ringer homogenate. Centrifuging for 5 min. at 18,000 r.p.m. in the refrigerator centrifuge lowers the rate of glycolysis somewhat, equally for free sugar and hexosediphosphate, but the apyrase is not preferentially inactivated.

Thus far we have been unable to prove this inactivation experimentally. When the homogenate is made in doubly distilled water with strong cooling and then tested in the presence of Mg, bicarbonate and ATP, its apyrase activity is the same as that shown in Fig. 1. Nevertheless, we believe that with longer exposure to water, higher temperature, greater dilution and other procedures detrimental to unstable

enzymes, such a gradual inactivation would be found, more pronounced in this case than in the presence of Ringer bicarbonate solution.

#### 4. TURNOVER OF HEXOSEDIPHOSPHATE

If all other glycolytic enzymes were present in excess, the activity of apyrase in homogenate would allow a  $Q_{La}$  of 200 for HDP. This condition is not fulfilled, but the  $Q_{La}$  for HDP amounts here to 45–50, in contrast to  $Q_{La}$  for glucose  $< 10$ . On the other hand, in the centrifuged extract only enough apyrase is left to give a rate for HDP =  $Q_{La}$  15–20. This expectation is fulfilled. After correcting for the blank, the  $Q_{La}$  in the extract is found to be around 15–20 for HDP, while glucose and fructose show  $Q$  values of 50 or higher. It is important that HDP is purified and freed from hexosemonophosphate, because the latter shows intermediate values between free sugar and HDP.

Two experiments are reproduced in Figs. 2 and 3. In the experiment of Fig. 2, 0.3 cc. of extract (1:5) was used, either alone, or with addition of the structural elements washed twice in diluted bicarbonate solution. The extract alone gives  $Q_{La}$  36 for fructose and  $Q_{La}$  29 for HDP. The extract + washed particles gives  $Q_{La}$  2 for fructose, about the same as the blank, and  $Q_{La}$  55 for HDP. The fundamental features are demonstrated quite clearly, although here, because the extract was made with bicarbonate without Ringer solution, more apyrase than usual passes into solution and the extract alone shows only small differences between HDP and fructose. The turnover of HDP in the usual Ringer homogenate and extract is shown in Fig. 3. HDP in the complete homogenate gives  $Q_{La}$  45 and in the extract 15.5. This difference, which is exactly the reverse of the rates of glycolysis of the free sugars (*cf.* 6), is due to the same cause, the distribution of the apyrase: HDP functions only as P-donor, and the quicker ATP is dephosphorylated, the faster the acylphosphate of 1,3-diphosphoglyceric acid can be discharged to the adenylic system. Free sugar functions only as P-acceptor. Its rapid phosphorylation requires the presence of a high concentration of ATP. As is shown in the preceding section, the normally added amount of ATP, 50  $\gamma$  pyro-P/0.3 cc. homogenate, loses its first and most reactive P-group in half a minute. Although all other transphosphorylations are the same and the intermediary steps are identical in the glycolysis of HDP and free sugar, their quite different and opposite rates in homogenate and extract are accounted for by the distribution of the

apyrase. In the living cell the activity of this enzyme is doubtless so adjusted to the activity of the other enzymes of the glycolytic cycle as to keep phosphorylation and dephosphorylation in step. *This co-ordination breaks down on disintegration of the tissue.*

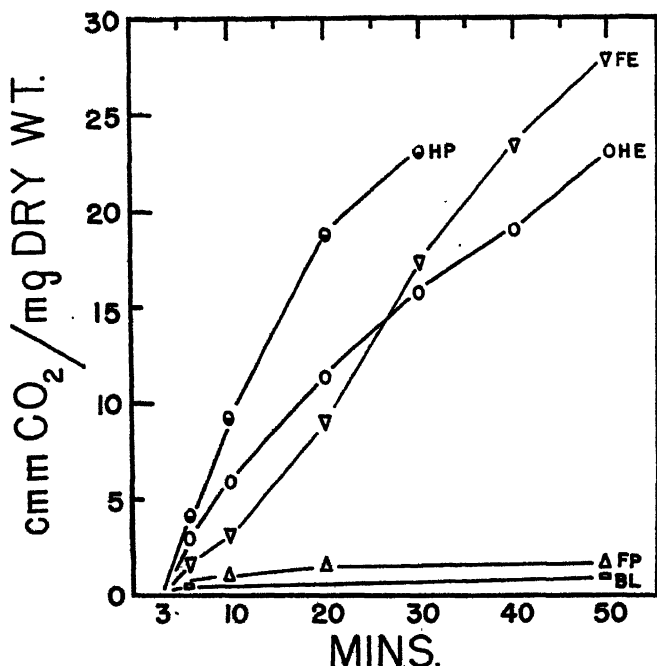


FIG. 2. Glycolysis in Extract (E) and Mixture of Extract and Washed Particles (P) (exp. 255). Homogenate made in  $4 \times 10^{-2} M$  bicarbonate + Mg and nicotinamide. Particles washed twice in  $4 \times 10^{-2} M$   $\text{NaHCO}_3$ , filled to the original vol. 0.2 cc. particles + 0.3 cc. extract taken in (P). Incubation in  $3 \times 10^{-2} M$  bicarbonate with  $5 \times 10^{-3}$  phosphate. At time 0 min. 0.1 cc. cozymase (0.3 mg. DPN) + 0.1 cc. ATP (45  $\gamma$  pyro-P) tipped in. Total vol. 1.0 cc.  $\square$  BL. Blank of extract.  $\nabla$  FE: 4 mg. fructose in extract.  $\Delta$  FP: 4 mg. fructose in extract + particles.  $\circ$  HE: HDP in extract (600  $\gamma$  P).  $\odot$  HP: the same in extract + particles.

The effect of arsenate is fully in line with the explanation given here. Arsenate allows HDP to be fermented at the same speed as sugar, even in the absence of apyrase. The mechanism of this reaction was explained formerly (10). As is shown in Fig. 3, arsenate ( $1 \times 10^{-3} M$ ) does not increase the turnover rate of HDP in the homogenate, but it

does so in the extract. In the former case the speed is not controlled by the apyrase, because this enzyme is far in excess, but in the latter case it depends on the apyrase; here arsenate is effective in allowing phosphopyruvic acid to be dephosphorylated in the absence of apyrase.

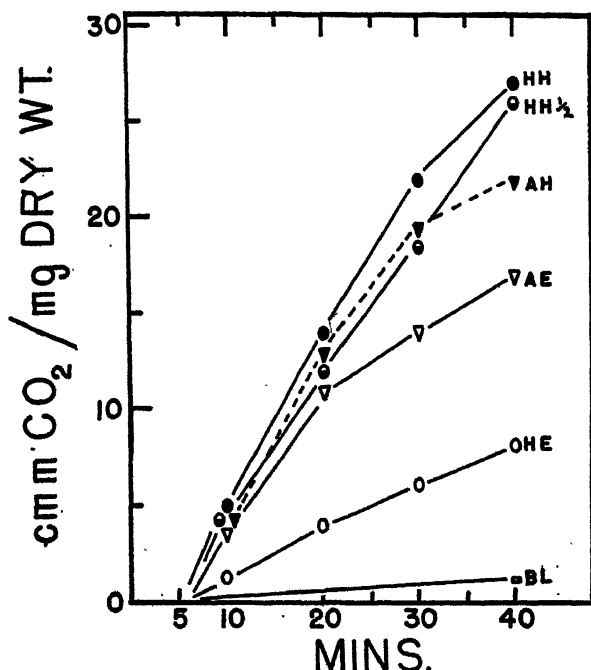


FIG. 3. Glycolysis of HDP in Ringer Homogenate and Extract (0.3 cc.) (exp. 268). Homogenate 1:5 with Ringer-Mg-bicarbonate. Same additions as in Fig. 2.  $\square$  BL: Blank of homogenate and extract.  $\bullet$  HH: HDP with 450  $\gamma$  P in homogenate.  $\circ$  HH  $\frac{1}{2}$ : same amount of HDP with 0.15 cc. homogenate.  $\nabla$  AH: dotted line HDP +  $1 \times 10^{-3}$  M arsenate in homogenate.  $\circ$  HE: HDP in extract.  $\nabla$  AE: the same +  $1 \times 10^{-3}$  M arsenate.

Still another factor is involved in the rate of glycolysis of brain tissue; the system creatine—CP as acceptor and donor of P. The concentration of creatine in rat brain, which amounts to 1.5 mg./g. of fresh tissue according to Kerr (11) and Gerard and Tupikowa (12), makes possible the formation of 360  $\gamma$  P of CP. In this way the store of energy-rich phosphate groups can be trebled, because 180  $\gamma$  pyro-P of ATP are



contained in 1 g. of brain. The equilibrium between the adenylic system and creatine is dependent on many factors, especially pH (*cf.* 13). In general, it can be said that free creatine will increase the rate of turnover of HDP in a low range of apyrase activity. Phosphocreatine will tend to raise the rate of turnover of free sugar in a low range of ATP concentration because it will rephosphorylate ADP. The first consequence was formerly demonstrated (1) with the help of dialyzed extract of rabbit brain, the second consequence, increased turnover of glucose in the presence of CP, was found by Geiger (14) in dialyzed extracts from rat brain. In the present work some such experiments were made with homogenates and extracts which confirmed our expectations. Because the brain suspensions are diluted 15–30 times as compared with the living tissue, the concentration of preformed creatine is low and CP is absent. Addition of creatine in amounts comparable with the concentration *in vivo* increased only the lactic acid formation from HDP in the centrifuged extract and inhibited in all other combinations. On the other hand, CP increased enormously the glycolysis of free sugars in the homogenate but had a small inhibiting influence in extracts. Indeed, the effect of CP in the homogenate is identical with that of repeated additions of ATP, as described in the preceding paper. Because the manometric experiments are vitiated by the change of basic equivalents during splitting and synthesis of CP, the lactic acid was determined chemically.

In Table I 0.5 cc. of homogenate (1:5) or extract were used in 1.6 cc. volume. The additions corresponded to those of the manometric experiments.

##### 5. SIMULTANEOUS TURNOVER OF FREE SUGAR AND HEXOSEDIPHOSPHATE

Since apyrase has opposite effects on glycolysis of free sugar and HDP, the best balanced system for use in securing a high and approximately constant turnover rate, independently of the amount of enzyme present, consists of a mixture of glucose and HDP. It was shown some time ago (8, 15) that, in fermentation or glycolysis systems which contained the enzymes of oxidation-reduction and some transphosphorylation enzymes, but were lacking in others and also in apyrase, a mixture of equivalent amounts of glucose and hexosediphos-

TABLE I  
*Glycolysis in the Presence of the System Creatine-CP*  
 30 min. 38°C.

I. ATP with 75  $\gamma$  pyro-P and 0.5 mg. DPN added in all samples.

II. ATP with 50  $\gamma$  pyro-P and 0.7 mg. DPN added in all samples.

Homogenate or extract H or E	Sugar	Creatine M	CP M	Lactic acid formed above blank	
				I	II
E	HDP*	0.015	0.01	198	228
E	HDP			253	315
E	glucose**				593
E	glucose				335
H	HDP	0.015	0.01	570	
H	HDP			435	
H	glucose			96	70
H	glucose			718	588
H	fructose	0.01	0.01		18
H	fructose				520

\* HDP:600  $\gamma$  P

\*\* glucose and fructose:4 mg.

phate reacted stoichiometrically with great speed. This has some bearing on the equation of Harden and Young (7). In the more complete glycolytic system, like the homogenate of brain, where stoichiometric relations are ruled out by the further reaction of the end products, such a combination of free sugar and HDP also reacts much better than the single constituents alone. This was found empirically by various authors who were mainly concerned with obtaining high and constant rates of glycolysis independently of the mechanism. Indeed, this also furnishes the clue to the results of Utter *et al.* (5) which we had ascribed in our preceding paper exclusively to the use of distilled water for preparation of the homogenate and the destruction of the apyrase. We had overlooked that the authors had not only "primed" the reaction by a trace of HDP (as we did ourselves), but had added to every sample 2.5 mM/cc., from which alone 0.45 mg. lactic acid or 112 mm<sup>3</sup>. CO<sub>2</sub> can be formed. It is evident from their data (*cf.* Figs. 3 and 4, p. 206 and 207) that for the first 30 min. nearly all of the lactic acid comes from this source, and that only later does the glucose turnover prevail. A similar experiment is reproduced here in Fig. 4. Homogenate 0.3 cc. in 1.3 cc.

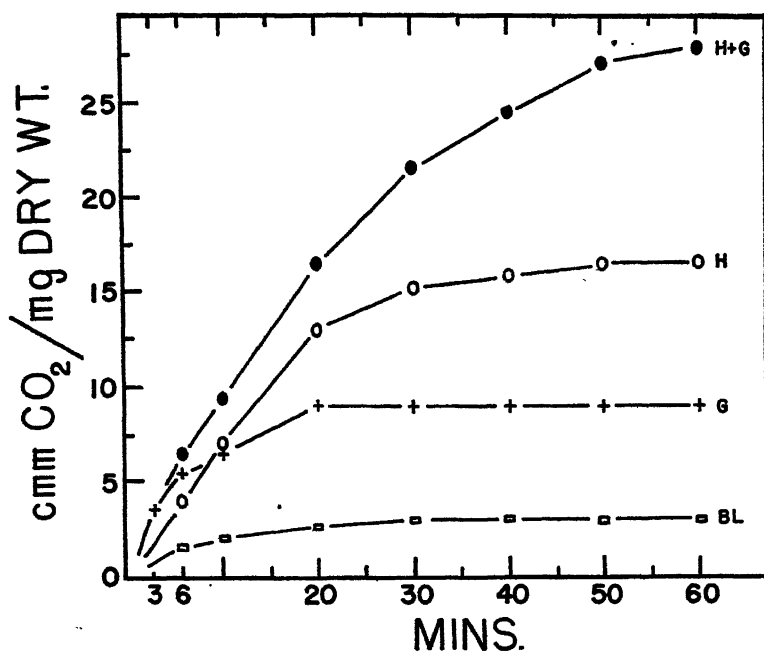


FIG. 4. Glycolysis of the System Glucose + Hexosediphosphate in Ringer-Homogenate (exp. 272). 0.3 cc. homogenate (10 mg. dry weight) in 1.3 cc. total volume. Same additions as in Fig. 2 and 3.  $\square$  BL. Blank.  $+$  G 4 mg. glucose.  $\circ$  H: HDP with 180  $\gamma$  P (= 135 mm<sup>3</sup>. CO<sub>2</sub>).  $\bullet$  H + G: 4 mg. glucose + 200  $\gamma$  P-HDP.

total volume was used (10 mg. dry weight). The amount of HDP added (180  $\gamma$  P or 3.0 mM) allowed a formation of 135 mm<sup>3</sup>. CO<sub>2</sub>. When the blank is subtracted, this is actually formed where HDP alone is present (curve H); the reaction is nearly completed in 30 min. With glucose (4 mg.) and a "trace" of HDP (0.15 mM equal to 7 mm<sup>3</sup>. CO<sub>2</sub>) the reaction stops after 20 min. This is due to the destruction of ATP, as was shown in the preceding paper. When both are present (curve H + G) the reaction, although slowly declining, keeps on for an hour. Similar curves are obtained with larger amounts of HDP + glucose or fructose, *e.g.*, experiment 271:

mm <sup>3</sup> . CO <sub>2</sub> formed in 1 hour:	blank	43
	HDP (550 $\gamma$ P)	342
	Fructose (4 mg.)	51
	HDP + fructose	430

Fructose, which alone gives only 8 mm<sup>3</sup>. more than the blank, increases the glycolysis of HDP by 88 mm<sup>3</sup>. and the curves follow the pattern of Fig. 4. However, one must bear in mind that these conditions are different from those in the living cell. HDP is not present in the cell in such high concentrations. On the other hand, hexosemonophosphate, which can function as P donor and acceptor, may play a role similar to that of a mixture of glucose and HDP. Moreover, the organization of the cell surely secures the coordination of the single enzymatic reactions by other means.

### DISCUSSION

The main topics were discussed in the preceding paper. The role of HDP as intermediate of glycolysis does not need further elaboration. Geiger (14), who contributed to our knowledge of the glycolysis of brain extract, was curiously led astray by the inability of his solutions to metabolize HDP into believing that this ester was not on the pathway of sugar breakdown. Actually his extracts were devoid of apyrase. Utter *et al.* (5) assumed that the "inhibitor" of glycolysis observed by Geiger in the structural elements, was mainly the nucleotidase, decomposing cozymase. While this undoubtedly contributes to the "inhibition" it is easy to show that, by checking this destruction with the help of nicotinamide, which we did in conformity with other authors, the inhibiting influence of the particles on glucose turnover is not removed. A second most potent "inhibitor" is the great amount of apyrase and unspecific phosphatases bound to the structures. But even if this is taken into account, there still remain other controlling factors which regulate the reactivities of the single hexoses toward ATP in the presence of adsorbed hexokinase.

We are happy to dedicate this paper to Professor Carl Neuberg who, as a pioneer in an early phase, has contributed so much to the understanding of the intermediary metabolism of carbohydrates.

### SUMMARY

The activity of adenylypyrophosphatase ("apyrase") in brain homogenate is compared with the turnover of hexosediphosphate (HDP). Nine-tenths of the apyrase activity of the homogenate is bound to the structural elements and centrifuged out, if the extract is prepared. Accordingly, the turnover of HDP is high in the homogenate and much lower in the extract, while, for the free sugars, the opposite is true.

The systems sugar + creatinephosphate and sugar + HDP, where P acceptor and donor activities are balanced, can increase and stabilize the lactic acid formation in brain homogenates as compared with sugar alone.

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# On the Enzymatic Destruction of Thiamine<sup>1</sup>

K. P. Jacobsohn and M. D. Azevedo

*From the Institute Rocha Cabral, Lisbon, Portugal*

Received February 24, 1947

## INTRODUCTION

Green, Carlson and Evans (1) observed that the chasteoparalysis of foxes, caused by feeding raw carp, could be prevented or cured by thiamine. Woolley (2) demonstrated that carp tissue contained a heat-labile substance which would inactivate thiamine *in vitro*, and Krampitz and Woolley (3) discovered the enzymic nature of the hydrolysis of thiamine, which was proved by demonstration of fragments produced from thiamine by the action of the tissue. These products have been identified as 4-methyl-5-hydroxyethylthiazole and 2-methyl-4-amino-5-hydroxymethylpyrimidine.

The same authors found that the reaction was but slightly affected by changes in pH in the range 1 to 8 and by changes in temperature from 0° to 37°C. The enzyme has been obtained in solution in 10% NaCl in good yield, and has been shown to consist of a heat-labile, non-dialyzable part and a heat-stable, dialyzable component.

To explore the physiological significance of the enzyme we have studied in preceding papers (4) its diffusion in animal and vegetable tissues and also its specific properties. We found (4) that this biocatalyst exists not only in the viscera of carp—which are rare in this country—but also in those of other fresh-water fishes of the *Cyprinidae* family. But the tissue of other cold-blooded animals and of the guinea pig have not destroyed thiamine, neither did the vegetables we have studied.

Further we have established a remarkable resistance against the toxic action of mercury ion, toluene and morphine, and acetylcholine also does not inhibit the enzyme activity.

In the present paper we have pursued our investigations on the diffusion of this enzyme and have compared the activity of several enzymic

<sup>1</sup> Dedicated to Professor Carl Neuberg on his 70th birthday.

preparations. The experiments outlined below indicate that the tissue of shrimps and some mussels are rich in this enzyme while crabs and some salt-water fish do not contain it. On the other hand, we show that extraction of the enzyme from the viscera can be carried out without the 10% NaCl solution used by Krampitz and Woolley; the extraction liquid can be diluted, and *M*/15 phosphate (pH: 6, 9) can also be used efficiently. Further, we have obtained from *Carassius* viscera a dry acetone preparation of the enzyme which is almost soluble in water and which does not adsorb thiamine as fresh tissue often does.

## EXPERIMENTAL

### *Preparation of Enzyme*

The viscera, removed immediately after the fish were killed, or the whole animal tissue, in the case of crustacea and mussels, were ground with 3 volumes of a 10% solution of NaCl or with physiological serum or with *M*/15 phosphate (pH = 6.9) and the suspensions allowed to stand over night at 15°C. and then centrifuged. The active extracts were stable for some weeks, but sometimes have shown a distinct adsorption of the vitamin (4) which often was not detectable. Further, we have obtained an active dry powder by treating one volume of the extract with 10 volumes of acetone. The precipitate was filtered by suction and washed quickly with acetone and ether. The dry preparation thus obtained was active and did not adsorb vitamin, 30 ml. of enzymic liquid corresponding to 0.42 g. of dry powder which was almost soluble in water.

### *Reaction Mixtures*

The enzymic liquids were incubated at 37°C. for several hours with varying amounts of thiamine (Benervé-Roche), water and a few drops of toluene. The vitamin-destroying potency of the different extracts, the composition of each reaction mixture and other experimental details are shown in the tables below.

### *Determination of Thiamine*

The activity of the preparations was determined by evaluating the velocity of thiamine fermentation. The remaining vitamin was estimated by the chemical method of the Jansen-school, based on the thiochrome reaction. Eight ml. of fermentation mixture were agitated with 1 ml. of 30% sodium hydroxide,  $\frac{1}{2}$  minute later 1 ml. of potassium ferricyanide solution (1%) was added and the thiochrome, formed by the oxidation of aneurin, extracted from the aqueous mixture by agitating with 10 ml. of amyl alcohol.<sup>2</sup> The two liquid layers were separated by centrifuging for 8

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<sup>2</sup> We had no isobutanol or franconite at our disposal, but amyl alcohol can also be used, although the intensity of the fluorescence is less.

minutes and the alcoholic layer decanted, dried with anhydrous sodium sulfate and filtered. The fluorescence of the liquid obtained was compared with those of a blank assay, in which the extraction was effected in the same way with an aqueous solution containing 25-75  $\gamma$  of thiamine (Table I, h; Table II, e).

TABLE I

## Reaction mixtures:

 Solution of thiamine (10 $\gamma$ /ml.)

Extract in 10% NaCl (a-g)

Water

2.5 ml. } 37°C.;

3 ml. }

2.5 ml. } pH 6.9

## Fermentation

fluorescence* compared with (a)	Before	After 4 hours
(a) <i>Carassius Carassius</i>	xx	—
(b) <i>Dentex Macrophthalmus</i>	xxx	xxx
(c) <i>Pagellus acarne</i>	xxx	xxx
(d) <i>Portunus marmoreus</i>	xxx	xxx
(e) <i>Penaeus Caramote</i>	xxx	—
(f) <i>Cardium edule</i>	xxx	xxx
(g) <i>Tellinae</i>	xxx	—
(h) Water	xxx	

TABLE II

## Reaction mixtures:

 Solution of thiamine (30 $\gamma$ /ml.)

*Carassius Carassius* extract (a-d)

Water

2.5 ml. } 37°C.;

3 ml. }

2.5 ml. } pH 6.9

## Fermentation

fluorescence* compared with (e)	Before	After 1 hour	After 3 hours
(a) 10% NaCl	xxx	x	—
(b) Physiological serum	xx	x	—
(c) M/15 phosphate	xxx	—	—
(d) Dry extract (0.14 g./7 ml.)	xxx	xx	x
(e) Water	xxx		

\* Not having a Cohen fluorometer at our disposal, we have used only the Philips Philora lamp, and have indicated the intensity of fluorescence of the "blank" with three crosses (xxx) and the lesser intensities with two ( $\pm 70\%$ ) or one ( $\pm 35\%$ ), a complete lack of fluorescence being indicated by a dash (—).



We have indicated in the tables, in addition to the fluorescence values observed after a fermentation of several hours, those found in the presence of thiamine at the beginning, especially because of the adsorption effect we have mentioned in a preceding paper. We have found (4) that sometimes this effect can be avoided by allowing the reaction mixture to stand for  $\frac{1}{2}$  hour with 5 ml. of trichloroacetic acid (5%) and neutralizing before the treatment with strong alkali.

### RESULTS

In Table I are recorded the activity of shrimp, *Carassius* and *Tellinae* extracts, while the others were inactive. On the other hand, the activity of our dry preparation is remarkable as well as the fact that it does not adsorb thiamine. Table II proves that the *Carassius* extract obtained with physiological serum adsorbs some of the vitamin and that its activity is a little inferior to that of the other preparations, the phosphate extract and the 10% NaCl extract being of the same efficacy.

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# The Estimation of Lipoxidase Activity<sup>1,2</sup>

James B. Sumner and Grant Newey Smith

*From the Biochemistry Laboratory, New York State College  
of Agriculture, Ithaca, New York*

Received January 27, 1947

## INTRODUCTION

In 1928 a carotene-destroying enzyme was found by Bohn and Haas (4) to occur in the soy bean, and this was employed to bleach dough made from unbleached wheat flour. In 1932 André and Hou (1) discovered that the soy bean contained an enzyme which oxidized unsaturated fats. They called this lipoxidase. In 1940 Sumner and Sumner (11) demonstrated that when lipoxidase peroxidizes an unsaturated fat, some highly reactive intermediate is produced which can bring about the oxidation of carotenoids or other plant pigments which may be present. Hence lipoxidase is identical with the enzyme of Bohn and Haas.

Various methods have been employed for the determination of lipoxidase activity. These methods can be divided into three classes: 1, those which employ the Warburg apparatus and measure the absorption of oxygen during the oxidation of the unsaturated compounds (6, 7, 15, 16); 2, methods in which the peroxides formed are estimated (2, 3, 8, 10, 13, 14, 15, 17); and 3, methods based on the decolorization of a carotene suspension containing unsaturated fat (2, 9, 11, 12). All of these methods possess certain disadvantages, as has been pointed out by Cosby and Sumner (5).

Methods which follow the decolorization of carotene have the drawback that carotene solutions are unstable and also that the carotene soon separates from the aqueous digests. We have replaced carotene with bixin, since this carotenoid is relatively stable, has about 5-fold more coloring power than an equal quantity of carotene (90%  $\beta$  caro-

<sup>1</sup> Dedicated to Professor Carl Neuberg on his 70th birthday.

<sup>2</sup> We wish to express our gratitude to the Rockefeller Foundation for financial assistance.

tene and 10%  $\alpha$ ), although it is decolorized at the same rate as carotene, and also because bixin does not separate from aqueous solutions as rapidly as carotene.

### PROCEDURE

Place in a clean dry test tube (18  $\times$  150 mm.) 0.1 ml. of neutralized fatty acid solution. Add 0.5 ml. of the stock bixin solution; then mix and add 5 ml. of gum arabic-phosphate buffer. Place in a constant temperature bath at 25 C. After the suspension has reached constant temperature, add from 0.1 to 1.0 ml. of properly diluted lipoxidase solution, gently mix the digest and start a stop watch. Determine by visual comparison the time necessary to bleach the suspension to the same intensity of color as that of a standard solution. This standard solution is prepared by mixing 0.1 ml. of the fatty acid solution, 0.1 ml. of the stock bixin solution, 5 ml. of the gum arabic-phosphate buffer and sufficient water to bring to the same volume as the solutions to be compared with it. If the enzyme solution imparts a turbidity to the solution, it may be necessary in some cases to add an equal volume of heat-inactivated enzyme suspension to the standard solution to aid in comparisons.

It is possible to employ a photoelectric colorimeter instead of the unaided eye. Here one determines the colorimetric reading of the standard solution, using a green filter. The unknown digest (at 25°–30°C.) is placed at once in a second colorimeter tube and the time required to bring the colorimeter reading of this to the reading of the standard is measured.

To measure the rate of decolorization of the bixin one can stop the reaction after various periods of time through the addition of 5 ml. of 7% sodium hydroxide. The solution can then be read in a photoelectric colorimeter, using a green filter. When one plots the rate of decolorization of bixin against time, a sigmoid curve is obtained (Fig. 1). This shows that it is advisable to carry out the determinations over that

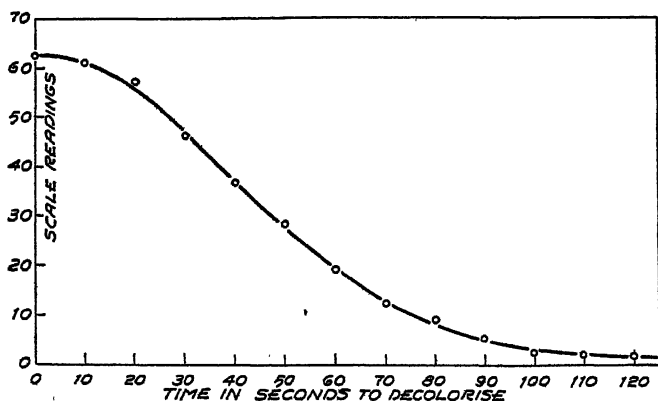


FIG. 1. Rate of Decolorization of Bixin by Lipoxidase.

portion of the curve which represents more or less a straight line. In this laboratory 80% decolorization has been selected as the end point of the determination, as it allows the maximum time to study the reaction with a high degree of accuracy.

### FACTORS AFFECTING THE REPRODUCIBILITY OF THE METHOD

The reproducibility of the method depends largely on taking a few fundamental precautions. Since this method depends on measurement of the intensity of light transmitted, a clear, homogeneous solution must be employed. If the various components are mixed according to the following order: fatty acid, bixin, gum arabic-phosphate buffer, enzyme, a solution with minimum turbidity is obtained which is suitable for use with a photoelectric colorimeter.

In mixing the components, care must be exercised not to cause inactivation of the lipoxidase, or the formation of a foam. This can be accomplished by slowly inverting the tube twice, after addition of the enzyme.

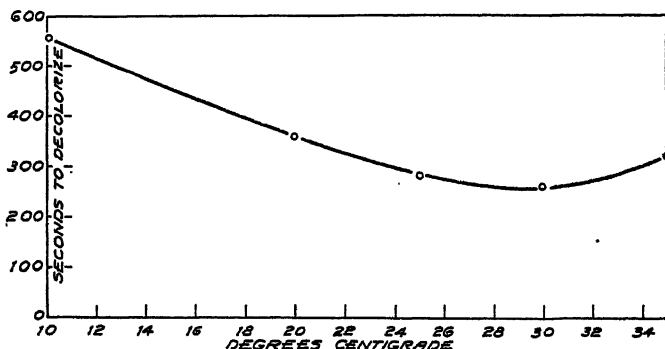


Fig. 2. Effect of Temperature on the Rate of Decolorization of Bixin.

The effect of increasing the temperature on the time of decolorization varies but little between 25° and 30°C. as is shown in Fig. 2.

We have run our tests at pH 6.5 which Sumner and Dounce (10) found to be the optimum for lipoxidase activity.

### RESULTS

The present method has been used in a considerable number of studies on purification and inactivation of lipoxidase preparations. The results of several of these determinations of lipoxidase activity are given in Table I. These figures were selected at random from a series of experiments in which the quantity of 40-fold purified soy lipoxidase

TABLE I

Sample	Time	Scale Reading	Time	Scale Reading
	<i>sec.</i>		<i>sec.</i>	
I	0	85.0	0	85.0
II	20	46.2	20	46.1
III	40	35.4	40	35.5
IV	60	31.0	60	31.0

employed was from 10 to 150  $\gamma$ , and they demonstrate the reproducibility of results by our method. The time is an indication of how long the reaction was allowed to proceed before the enzyme was inactivated with sodium hydroxide, and the quantity of bixin remaining in the solution determined by use of a Fischer photoelectric colorimeter.

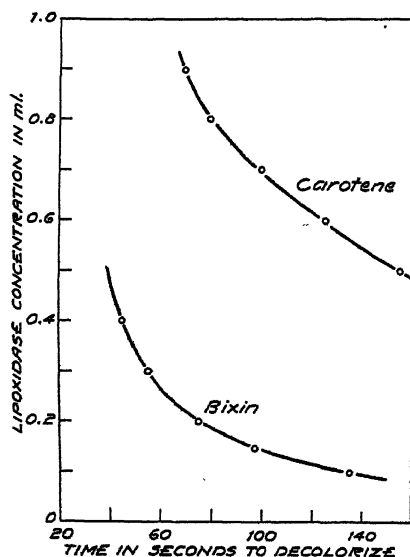


FIG. 3. Comparison of the Decolorization of Bixin and Carotene.

The method was also compared with the standard carotene decolorization method (11) which had previously been employed in this laboratory (see Fig. 3). Here equal concentrations of both carotene and bixin were employed.

## REAGENTS

*Purified Lipoxidase*

Stir 100 g. of finely ground defatted soy bean meal with 500 ml. of ice cold water in the cold room. Add with stirring, 10 ml. of 2 *N* acetic acid. Filter rapidly in the cold room. As fast as the filtrate drips into the receiving vessel, add 0.5 *N* disodium phosphate, using a total of about 50 ml. for about 250 ml. of filtrate.

*Fatty Acid Solution*

Fat was extracted from soy bean meal with petroleum ether, and after evaporating the petroleum ether 50 g. of the resulting soy bean oil were saponified with alcoholic sodium hydroxide. After the saponification was completed, the alcohol was evaporated and water added. The free fatty acids were liberated from the soap solution by the addition of 10% sulfuric acid, collected and washed several times with hot distilled water. The free fatty acids were then stored in brown bottles in the ice chest until needed for the determinations. This material has been found to remain free from peroxides for more than a year.

The fatty acid solution used in the assay was prepared by dissolving 1.0 ml. of the melted fatty acids in 100 ml. of 95% alcohol. The solution was neutralized with 0.1 *N* sodium hydroxide, using phenolphthalein as indicator. The solution was then diluted to 300 ml., using 95% alcohol. This solution must not be alkaline.

*Bixin Solution*

The bixin solution was prepared by dissolving crystalline bixin in 95% alcohol so that the final concentration was 0.02 mg./ml. of solution. A few drops of 0.1 *N* sodium hydroxide were added to aid in dissolving the bixin.

The crystalline bixin was prepared by extracting 100 g. of annatto powder (Fisher Scientific Co.) with 2 liters of boiling acetone. The solution was filtered hot and this filtrate evaporated to about 200 ml. and allowed to stand overnight at room temperature, during which the bixin precipitated out in crystalline form. The crystals were washed several times with absolute alcohol. They were again recrystallized from boiling acetone, washed with absolute alcohol, and dried in a desiccator. The melting point (uncorr.) was 196–198°C.

*Gum Arabic-Phosphate Buffer*

Forty g. of gum arabic were added to 500 ml. of distilled water and heated on a steam bath until completely dissolved. The solution was brought to about pH 6.5 by addition of approximately 20 ml. of 0.1 *N* sodium hydroxide. To this was added 200 ml. of 0.5 *M* phosphate buffer pH 6.5. The solution was heated on the steam bath for one hour and then the calcium phosphate that had formed was filtered off overnight. The filtrate was diluted to 2 liters with distilled water and a few drops of toluene added as preservative. This solution was kept in the ice chest while not in use.

The 0.5 *M* phosphate buffer of pH 6.5 was prepared by mixing 60 ml. of 0.5 *M*  $\text{KH}_2\text{PO}_4$  and 40 ml. of 0.5 *M*  $\text{Na}_2\text{HPO}_4$ .

## SUMMARY

A method for estimation of lipoxidase is described which is believed to be more satisfactory than previous methods.

The carotenoid bixin is employed in place of the carotene which had previously been used to determine lipoxidase activity. Bixin has the advantages that it can be easily obtained in the crystalline form, it is relatively stable, has about 5-fold more coloring power than carotene, does not separate rapidly from aqueous solutions, and is decolorized rapidly when used in the method described above.

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# Inositol Content of Blood Plasma<sup>1</sup>

Sulamith Sonne and Harry Sobotka

*From the Division of Chemistry, Laboratories, and the Neurological  
Service of the Mount Sinai Hospital, New York City*

Received February 10, 1947

## INTRODUCTION

The development of micro bioassay methods for inositol has made it possible to determine this substance in grains and other food stuffs, yeast, animal organs, *etc.*, the content of which is of interest because of the vitamin functions of this cyclic carbohydrate (1). We have adapted the method of Woolley, with the modifications by Atkin (2), to the determination of the inositol content of blood plasma. The procedure consists in a nephelometric evaluation of the growth of a strain (No. 4228) of *Saccharomyces carlsbergensis* after 16–17 hours in a medium supplying all necessary growth factors except inositol. Various details of the method and of the recovery checks, carried out with each test, are described in the Experimental Part.

The range of inositol content in plasma from individual subjects was 0.42–0.76 mg./100 ml. A wider range was found in determinations on pooled plasma from miscellaneous patients: the values were 0.54, 0.65, 0.66, 0.69, 1.00, 1.03, 1.31, 1.32, 1.42, and 1.87 mg./100 ml.

In a number of patients suffering from amyotrophic lateral sclerosis, the values were between 0.37 and 0.67 mg./100 ml. These patients had been selected for inositol studies in view of a report (3) that per oral inositol administration exerted a favorable influence on the therapeutic effects, and thus, by implication, on the absorption and utilization of tocopherol in certain myopathies. No such therapeutic effects could be observed with the use of inositol as an addition to tocopherol in cases of amyotrophic lateral sclerosis; a clinical report will be given by Dr. I. S. Wechsler elsewhere.

One normal subject and four with amyotrophic lateral sclerosis were

<sup>1</sup> Dedicated to Professor Carl Neuberg on his 70th birthday.



given daily 1.50 g. of inositol for several weeks and the plasma level was analyzed at weekly intervals. The table summarizes the results which show moderate irregular increases except for case number 103.

TABLE I  
*Plasma Inositol Values In Normals And Patients Before And After Inositol Administration*

No.		Mg. inositol in 100 ml. plasma			
		Starting level	After administration of 1.5 g. inositol <i>per diem</i>		
			for 1 week	for 2 weeks	for 3 weeks
1	Normal	0.54	0.46	0.70	0.93
2	Normal	0.76	—	—	—
3	Normal	0.42	—	—	—
101	Amyotr. Lat. Scler.	0.62	1.04	1.11	—
102	Amyotr. Lat. Scler.	0.47	0.50	0.82	0.75
103	Amyotr. Lat. Scler.	0.67	0.52	0.33	—
104	Amyotr. Lat. Scler.	0.37	0.80	0.57	—
105	Amyotr. Lat. Scler.	0.45	—	—	—

Values obtained by this method in materials, other than blood plasma, have been accepted as true values in spite of the rigorous conditions of hydrolysis. The purpose of this hydrolysis is twofold: to liberate conjugated inositol and to destroy proteins, the presence of which would produce opacity during sterilization. In experiments not reported here, we have attempted to avoid these rigorous conditions for deproteinization of the plasma. In the course of these studies we have reached the conclusion that a considerable portion of the plasma inositol is non-conjugated and ultrafiltrable. Inositol seems to be partly destroyed, or masked, under essentially milder conditions of acidity and time than those used by Woolley and by Atkin. Hence, the plasma inositol values given represent a lower limit and the actual amount of inositol in plasma may be higher. The forms in which inositol occurs in plasma need further investigation.

#### EXPERIMENTAL

Individual blood specimens were obtained with potassium oxalate in the fasting state in the customary manner. Ten ml. of plasma were hydrolyzed with 100 ml. of 18% HCl for 6 hours and the hydrolyzate filtered through a sintered glass filter.

The filtrate was concentrated *in vacuo*, taken up in 30 ml. of distilled water, treated with a small amount of Norit, and after filtration, the volume was made up to 50 ml. The pH of the acid solution is brought to ca. 5.5 by a few drops of 10% NaOH. Usually, duplicate samples of 2, 3, and 4 ml. of this solution are placed in 50 ml. Erlenmeyer flasks, and 5 ml. of the medium are added. Finally, all volumes are made up to 9 ml. with distilled water.

The medium is prepared by combination of the following solutions:

Sugar and salts .....	500 ml.
Potassium citrate buffer .....	100 ml.
Ammonium sulfate (15%) .....	50 ml.
Calcium pantothenate (200 $\gamma$ /ml.) .....	25 ml.
Pyridoxine (10 $\gamma$ /ml.) .....	50 ml.
Thiamine (10 $\gamma$ /ml.) .....	50 ml.
Biotin (1 $\gamma$ /ml.) .....	50 ml.
Casein (acid hydrolyzed) 10% .....	100 ml.
Water .....	to 1000 ml.

*Sugar and Salts Solution.* One liter contains 200 g. of C.P. dextrose (anhydrous), 2.2 g. of monopotassium phosphate, 1.7 g. of potassium chloride, 0.5 g. of calcium chloride ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ), 0.5 g. of magnesium sulfate, 0.01 g. of ferric chloride, and 0.01 g. of manganese sulfate.

*Potassium Citrate Buffer.* One liter contains 100 g. of potassium citrate ( $\text{K}_3\text{C}_6\text{H}_5\text{O}_7 \cdot \text{H}_2\text{O}$ ) and 20 g. of citric acid ( $\text{H}_3\text{C}_6\text{H}_5\text{O}_7 \cdot \text{H}_2\text{O}$ ).

Sterilization, inoculation, and incubation are carried out according to Atkin (2) and the final nephelometric reading is performed in a Klett-Summerson photoelectric colorimeter with filter number 66.

In addition to the 6 flasks with aliquots of the unknown, 10 flasks are set up containing standard amounts of 1, 2, 4, 6, and 8- $\gamma$  inositol in duplicate, and also 4 flasks for recovery tests, two of them containing 2 ml. of the unknown + 4- $\gamma$  inositol and two with 3 ml. of the unknown + 2- $\gamma$  inositol. In 26 tests the recoveries at both levels averaged 90% with a standard deviation of 8%.

#### ACKNOWLEDGMENT

This investigation was supported by a grant from Hoffmann-LaRoche, Inc., to Dr. I. S. Wechsler, Neurologist to the Hospital, and Dr. H. Sobotka, Chemist to the Hospital.

#### SUMMARY

The application of the nephelometric micro bioassay with *Saccharomyces carlsbergensis* to the determination of inositol in human blood plasma is described.

The range of plasma inositol in normals and certain patients is 0.37–0.76 mg./100 ml. for individual fasting samples. Pooled plasma from miscellaneous patients ranged from 0.54–1.87 mg./100 ml.

Daily ingestion of 1.50 g. of inositol usually produces a moderate rise of the plasma inositol level.

Destruction of some inositol during acid hydrolysis cannot be excluded. The state of inositol in the plasma needs further investigation.

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# Stereochemical Configuration and Provitamin A Activity

## VI. Some *Cis-Trans* Isomers of $\gamma$ -Carotene<sup>1</sup>

Harry J. Deuel, Jr., Cornelia Hendrick, Evelyn Straub,  
A. Sandoval, J. H. Pinckard and L. Zechmeister

*From the Department of Biochemistry and Nutrition, University of Southern California,  
School of Medicine, Los Angeles, and  
The Gates and Crellin Laboratories of Chemistry, California Institute  
of Technology, Pasadena, California<sup>2</sup>*

Received November 4, 1946

### INTRODUCTION

In Part II of the present series (1), the provitamin A potencies of two members of the stereoisomeric  $\gamma$ -carotene set were investigated, namely, that of ordinary  $\gamma$ -carotene (all-*trans*- $\gamma$ -carotene, *ex Mimulus*),  $C_{40}H_{56}$ , and of a naturally occurring poly-*cis* compound, pro- $\gamma$ -carotene,  $C_{40}H_{56}$ . In the present paper we wish to report on some bioassays in which some additional members of the  $\gamma$ -carotene set were studied. These included the following: (a) all-*trans*- $\gamma$ -carotene. Since there is an unexplained difference between the melting points of various preparations of all-*trans*- $\gamma$ -carotene crystals (3) which may or may not demand in the future a revision of certain structural interpretations,<sup>3</sup> we repeated our experiments with a  $\gamma$ -carotene sample obtained from another source, namely the ripe berries of *Pyraecantha angustifolia* Schneid. (b) A *cis* isomer of  $\gamma$ -carotene, tentatively termed neo- $\gamma$ -carotene P. This compound was isolated as a well crystallized compound from the same fruit but does not occur to any marked amounts in those mixtures of stereoisomers which can be obtained by refluxing or iodine catalysis of ordinary  $\gamma$ -carotene. (c) "Mixed neo- $\gamma$ -carotenes"

<sup>1</sup> Dedicated to Professor Carl Neuberg on his 70th birthday.

<sup>2</sup> Contribution No. 1104.

<sup>3</sup> For example, it cannot be safely excluded at the present time that some of the lower-melting samples which were isolated from various plants (including *Pyraecantha*) might be a dihydro derivative in which the isolated double bond is saturated, or a mixture of  $\gamma$ -carotene and dihydro- $\gamma$ -carotene.

which consisted of the total stereoisomeric mixture appearing below the unchanged portion of all-*trans*- $\gamma$ -carotene on the Tswett column. While it is easy to isolate some individual *cis-trans* isomers of  $\alpha$ - or  $\beta$ -carotene on the chromatographic column in small scale experiments, as was reported earlier, the corresponding zones obtained from  $\gamma$ -carotene form a complicated mixture consisting mainly of several groups of stereoisomers which cannot be resolved reliably (2). Therefore, some bioassays were carried out with such mixtures of the neo-compounds.

#### EXPERIMENTAL AND RESULTS

The isolation of all-*trans*- $\gamma$ -carotene and its neo form "P" (m.p. 88.5–90°C.) from *Pyraacantha* berries will be described elsewhere. A few milligrams of the analyzed crystals were dissolved in peroxide-free absolute ether, evaporated in a stream of  $\text{CO}_2$  and dissolved in Wesson oil.

To obtain the "mixed neo- $\gamma$ -carotenes," 12 mg. of  $\gamma$ -carotene in 50 ml. of petroleum ether (b.p. 60–70°) was refluxed in an all-glass apparatus, in diffuse daylight for  $\frac{1}{2}$  hour while nitrogen bubbled through. The solution was then developed on a 22  $\times$  4.4

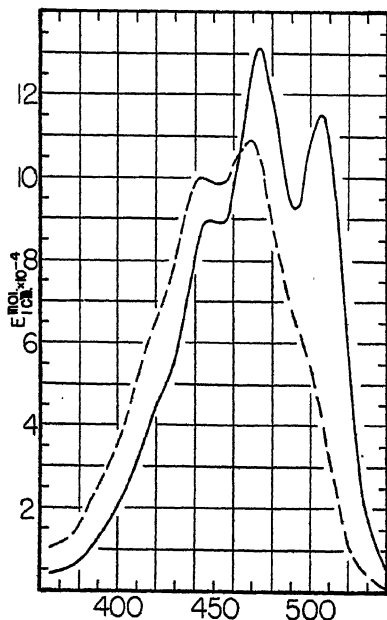


FIG. 1. Molecular Extinction Curves of All-*trans*- $\gamma$ -carotene (full line) and Pro- $\gamma$ -carotene (dashed line) in Wesson Oil. Wavelengths in  $m\mu$ .

cm. calcium hydroxide column (Arrowhead Lime Products, mixed with celite 3:1) with petroleum ether containing 2% acetone. Below the orange-red main zone of unchanged all-*trans* form, a heterogeneous, much lighter zone appeared which was eluted with methanol. This eluate was kept at 0°C. while the upper zone was re-isomerized and chromatographed. After 4 such isomerizations, the combined mixed neo- $\gamma$ -carotenes were transferred with water from methanol into petroleum ether and freed by chromatography from a small amount of all-*trans* contaminant. The main zone was then eluted with peroxide-free absolute ether and diluted to 150 ml. From this, 120 ml. was evaporated and dissolved in Wesson oil while three 10 ml. samples were necessary to estimate the concentration, as follows.

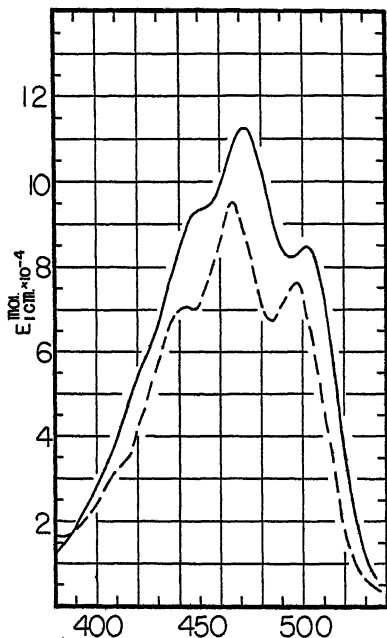


FIG. 2. Molecular Extinction Curves in Wesson Oil of Neo- $\gamma$ -carotene P (full line) and of Mixed Neo- $\gamma$ -carotenes (dashed line) Obtained by Refluxing of the All-*trans* Form and Having Decreased Adsorption Affinities. Wavelengths in  $m\mu$ .

Each of two such aliquots was evaporated, dissolved in 40 ml. of petroleum ether, refluxed for 30 min. as above, diluted to 100 ml. and estimated in the Beckman spectrophotometer on the basis of earlier data. The third 10 ml. aliquot, after evaporation, was dissolved in Wesson oil and extinction curves were taken (Figs. 1 and 2; see also Table I).

During the bioassay period the oil solutions were kept in dry ice, in darkness, and showed practically no change in the extinction values.

TABLE I

*Molecular Extinction Coefficients of Some Stereoisomeric  $\gamma$ -Carotenes in Wesson Oil at the Wave Length of Maximum Extinction*

Compound	Wave length (m $\mu$ )	E <sub>1 cm.</sub> <sup>mol.</sup> $\times 10^{-4}$
All-trans- $\gamma$ -carotene	473-4	13.1
Neo- $\gamma$ -carotene P	471-2	11.2
Mixed neo- $\gamma$ -carotenes	466-7	9.5
Pro- $\gamma$ -carotene	468-9	10.8

The bioassays were carried out according to the U.S.P. XII procedure and our earlier contributions, parallel with new tests of pure  $\beta$ -carotene as a standard. The levels fed daily (six times a week) were the following ones:  $\beta$ -carotene, 0.5 and 1.0  $\gamma$ ; all-trans- $\gamma$ -carotene, 2.4  $\gamma$ ; mixed neo- $\gamma$ -carotenes, 2.4 and 4.8  $\gamma$ ; neo- $\gamma$ -carotene P, 1.2 and 2.4  $\gamma$  (first series) and 0.5, 1.0 and 2.0  $\gamma$  (second series). The daily dosage was present in 0.1 ml. of Wesson oil which contained 0.5 mg. of  $\alpha$ -tocopherol. In all 212 rats of our stock colony were used in the two series of experiments which were carried out two months apart. The results of the bioassays are summarized in Tables II and III and illustrated in Fig. 3.

TABLE II

*Summary of Series I of Bioassay Experiments on Male and Female Rats Receiving All-trans- $\beta$ -carotene, All-trans- $\gamma$ -carotene, Mixed Neo- $\gamma$ -carotenes, Neo- $\gamma$ -carotene P in Cottonseed Oil or the Oil Alone (Negative Controls)*

(The average results on males and females are weighted equally. Where animals died in the course of the experiment, the number of animals still alive, which is included in the average, is given in parentheses. The average age at the start of the depletion period was 22-23 days.)

Carotene supplement	Dose per day	Number of rats		Depletion Period			Assay Period						
		Male	Female	Average weight at start	Average duration	Average final weight	Average increase in body weight up to the following days						Average final weight
							5th	10th	15th	20th	25th	28th	
All-trans- $\beta$ -carotene	$\gamma$			<i>g.</i>	<i>days</i>	<i>g.</i>	<i>g.</i>	<i>g.</i>	<i>g.</i>	<i>g.</i>	<i>g.</i>	<i>g.</i>	
	0.5	4	6	41.8	22	75.6	-0.9	7.0	13.7	17.7	21.2	23.9	99.5
All-trans- $\gamma$ -carotene	1.0	4	7	40.9	21	75.5	0.5	22.4	35.4	42.1	52.1	57.6	133.1
	2.4	5	7	41.6	22	74.8	1.4	9.7	16.4	22.7	27.7	31.2	106.0
Mixed neo- $\gamma$ -carotenes	2.4	5	6	41.5	21	75.3	-1.4	6.9	8.2	8.5	14.6	16.8	89.1
	4.8	5	6	41.8	21	75.5	3.2	11.7	20.9	27.6	(10) 35.2	(10) 39.2	114.6
Neo- $\gamma$ -carotene P	1.2	4	7	42.2	21	74.0	3.2	8.9	13.2	14.5	16.1	17.0	91.0
	2.4	5	7	42.0	22	74.4	3.7	10.1	15.2	21.6	26.5	26.0	100.4
	4.8	5	6	42.7	22	77.0	5.0	14.1	27.9	32.6	39.7	42.0	119.0
Negative controls	0.0	10	11	42.0	20	73.7	-2.2	-1.8 (18)	-2.2 (17)	-6.5 (13)	-10.1 (7)	-11.5 (4)	64.0

TABLE III

*Summary of Series II of Bioassay Experiments on Rats Receiving All-trans- $\beta$ -carotene or Neo- $\gamma$ -carotene P in Cottonseed Oil or the Oil Alone (Negative Controls)*

(The average results on males and females are weighted equally. Where animals died in the course of the experiment, the number of animals still alive, which is included in the average, is given in parentheses. The average age at the start of the depletion period was 22-23 days.)

Carotene supplement	Dose per day	Number of rats		Depletion Period			Assay Period						
		Male	Female	Average weight at start	Average duration	Average final weight	Average increase in body weight up to the following days						Average final weight
							5th	10th	15th	20th	25th	28th	
All-trans- $\beta$ -carotene	0.5	8	8	42.9	20	82.0	9.4	14.4 (15)	18.4 (15)	29.7 (15)	33.1 (15)	35.1 (15)	116.7
	1.0	9	8	43.2	19	81.5	9.3	22.2	35.1	45.6	50.9	53.3	134.8
Neo- $\gamma$ -carotene P	0.5	9	8	42.3	19	79.9	5.6	7.7	10.4	10.8 (16)	6.1 (16)	6.8 (14)	84.0
	1.0	9	8	43.3	20	81.4	2.8	6.9	13.2 (16)	12.8 (16)	13.8 (15)	13.2 (15)	94.6
	2.0	9	7	42.2	19	78.2	6.8	13.1	21.1	24.5	28.2	30.2	108.4
Negative controls	0.0	12	6	41.7	20	80.8	3.2	0.7 (17)	5.6 (16)	2.5 (15)	-5.0 (13)	-7.0 (12)	76.3

*$\beta$ -Carotene.* The average growth responses calculated for 0.8  $\gamma$  of  $\beta$ -carotene were 46.7 and 47.5 g. in the two series which give corrected values of 54.5 and 51.3 g., respectively, when allowance is made for the fact that this pigment was administered 24 instead of 28 days. The average growth response in 7 previous bioassays calculated for 0.8  $\gamma$  doses amounts to 43.5 g. The average slope of the growth curves for the two levels of  $\beta$ -carotene was 111.2 and 69.2 for the two series (previous average, 65.0).

*All-trans- $\gamma$ -Carotene.* The average gain in weight was 31.2 g., which gives a potency of 24.2% compared with  $\beta$ -carotene. This compares well with the value reported earlier for the same carotene obtained from *Mimulus*, which was 28%.

*Neo- $\gamma$ -Carotene P.* In the first series, the average growth of the rats receiving 2.4 or 4.8  $\gamma$  was 26.0 and 42.0 g. The potencies calculated for these values were 18.0 and 14.2%, respectively, of that of pure  $\beta$ -carotene. Because the slope of the curve for the rats receiving neo- $\gamma$ -carotene P was not quite parallel with that of  $\beta$ -carotene, a second confirmatory series was carried out. The average growth found was 6.8, 13.2 and 30.2 g. for the rats which received 0.5, 1.0 or 2.0  $\gamma$  respectively. The potency could be calculated only on the basis of the highest dose. This amounted to 20.6% of that of  $\beta$ -carotene.

*Mixed Neo- $\gamma$ -Carotenes.* The average growth at the levels of intake of 2.4 or 4.8  $\gamma$  daily was 16.8 and 39.2 g., respectively. The calculated potencies of these values are 18.0 and 14.2% of that of  $\beta$ -carotene. These results are given in Table IV.



TABLE IV

*Relative Provitamin A Activities of Some cis-trans Isomeric  $\gamma$ -carotenes*  
(The italicized figures denote the new results)

Compound	Potency in per cent of that of $\beta$ -carotene	Best value
All- <i>trans</i> - $\gamma$ -carotene	28, <i>24</i>	26
Neo- $\gamma$ -carotene P	<i>22, 15, 21</i>	19
Mixed neo- $\gamma$ -carotenes	<i>18, 14</i>	16
Pro- $\gamma$ -carotene	44	44

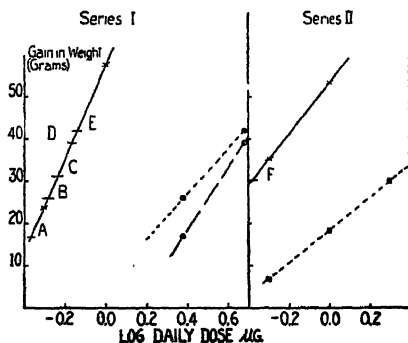


FIG. 3. Relationship of Gain in Weight to Log of Daily Dosage of  $\beta$ -Carotene, All-*trans*- $\gamma$ -carotene, Mixed Neo- $\gamma$ -carotenes and Neo- $\gamma$ -carotene P.

Solid lines represent the curves obtained for  $\beta$ -carotene, short dashed lines those for neo- $\gamma$ -carotene P and long dashed lines those for mixed neo- $\gamma$ -carotenes. Points A and D are the projections on the  $\beta$ -carotene curve of the weight increase of rats receiving daily 2.4 or 4.8  $\gamma$  of mixed neo- $\gamma$ -carotenes respectively. Points B and E are the projections of weight increase of rats receiving daily 2.4 and 4.8  $\gamma$  of neo- $\gamma$ -carotene P, respectively. Point C is the projection of weight increase of rats receiving daily 2.4  $\gamma$  of all-*trans*- $\gamma$ -carotene. Point F in the second series is the projection on the  $\beta$ -carotene curve of the weight increase of rats receiving daily 2.0  $\gamma$  of neo- $\gamma$ -carotene P.

### SUMMARY

The following provitamin A potencies of *cis-trans* isomeric carotenes were found in rats ( $\beta$ -carotene = 100%): all-*trans*- $\gamma$ -carotene, 26%; neo- $\gamma$ -carotene P, 19%; and mixed stereoisomers of  $\gamma$ -carotene (with decreased absorbability), 16%.

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# Yields and Vitamin Content of Food Yeasts Grown on Different Kinds of Molasses <sup>1,2</sup>

P. N. Agarwal, K. Singh, P. S. King and W. H. Peterson

From the Department of Biochemistry, College of Agriculture,  
University of Wisconsin, Madison

Received January 24, 1947

## INTRODUCTION

Beet molasses is the commonly used carbohydrate material for the production of bakers' yeast. Molasses from certain areas is preferred by some companies to that from other regions because of the higher yields obtained from the preferred molasses. Whether the apparent superiority of a given molasses is related to climatic conditions, methods of manufacture, differences in composition, or presence of toxic substances is not clear. A systematic study of the fermentability of different kinds of molasses has, therefore, been undertaken. This paper deals with the production of several new food yeasts in shaken flask cultures and the amounts of certain important B vitamins contained in these yeasts. Subsequent papers will deal with the production of yeasts under different conditions and also with the fermentability of different kinds of molasses by other industrial microorganisms.

The yeasts selected for study include a typical bakers' yeast, *Saccharomyces cerevisiae*, a strain of *Torula utilis*, and two yeasts, *Candida arborea* and *Oidium lactis*, that have been used in Germany for the production of food yeast. Several papers dealing with the production of these yeasts on various substrates have appeared. Sulphite waste liquor has been used in Germany (1, 2) for the production of *Torula* and *Oidium lactis*. From sulphite waste liquor obtained from 100 tons of completely dry wood, 7 tons of dry *Torula* yeast has been obtained by a continuous process (1). Its use as an inexpensive nutrient for livestock and poultry was suggested. From waste residues of alcohol fermentation, Peukert (2) obtained 60-100% yields, based on reducing sugar, of two fungi, *Biosyn* I and *Biosyn* II. The high yields of these

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<sup>1</sup> Dedicated to Professor Carl Neuberg on his 70th birthday.

<sup>2</sup> Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant-in-aid from the Sugar Research Foundation, Inc., New York.

yeasts were due to the fact that they utilized other carbon sources in addition to reducing sugars. The two materials were later reported (3) to be a mixture of *Oidium lactis*, *Fusarium aqueductum* and other types.

Production of yeast from wood sugar, obtained by either the Scholler or the Bergius process, has been extensively studied in Germany. Wood sugar has been used (16) for the production of some new food yeasts like *Torula utilis*, *Torula pulcherrima*, *Monilia candida*, *Candida arborea*, and mixed *Torula*. Using wood hydrolyzates, Fink and associates (4, 5, 6) obtained a 40% yield of *Torula utilis* based on reducing sugar. In feeding trials with farm animals it appeared to have a high nutritive value (7, 8).

Wood sugar has also been used in the United States for the production of fodder yeast. Peterson *et al.* (9) used hydrolyzates from 13 species of wood and 9 types of yeast. *Torula utilis* No. 3, *Candida tropicalis*, yeast P-13, later identified as *Mycotorula lipolytica* (10), and *Hansenula anomala* were the most satisfactory yeasts. Yields of 30–40%, based on reducing sugar, were obtained with *T. utilis*. Recently Kurth (10) and Kurth and Cheldelin (11) obtained 40–48% yields of *Torula utilis* No. 3, *Mycotorula lipolytica*, and *Hansenula suaveolens* Y-838, based on sugar present, from wood sugar stillage (residues remaining after alcoholic fermentation). They determined the B vitamins and amino acids contained in these yeasts and found good amounts of these compounds to be present.

Production of *Torula utilis* from molasses has been studied extensively by Thaysen and others (12) and a plant for its production from this material has been under construction in Jamaica. Using three different substrates, glucose-salt medium, molasses, and grain-wort, Pavcek *et al.* (13) obtained 30%, 35%, and 40% yields of commercial bakers' yeast respectively.

Fruit juice substrate has been used by Lewis *et al.* (14) who obtained a 53% yield of *Torulopsis utilis*, based on sugar present. With molasses as the substrate they obtained a 56% yield of the same yeast.

On asparagus juice medium and molasses medium Feustel and Humfeld (15) reported 46.2% and 45.8% yields of *Saccharomyces cerevisiae*, respectively, based on sugar supplied.

## EXPERIMENTAL

### *Yeast Strains*

Four strains of yeast, namely, *Saccharomyces cerevisiae* No. 53, *Torulopsis utilis* No. 3, *Candida arborea*, and *Oidium lactis* A were mainly studied.

Transplants of *Saccharomyces cerevisiae* and *Torula utilis* were obtained from Dr. Elizabeth McCoy of the Department of Agricultural Bacteriology. Cultures of *Candida arborea*, *Oidium lactis* A, and some other yeasts from Germany, notably *Candida pulcherrima*, *Monilia candida*, and other types described as *Predominant*, *Mycelial*, and *Lesser* were kindly sent to us through the courtesy of Mr. H. J. Bunker, Barclay, Perkins and Co. Ltd., London, and Dr. K. R. Butlin, Department of Scientific and Industrial Research, Chemical Research Laboratory, Teddington. A detailed discussion regarding the origins and use of these cultures in Germany is given in Fiat Final Report No. 499 (16).

Yeast transfers were made every two weeks on a molasses-agar medium.

### *Inoculum*

The medium for growing the inoculum was essentially that recommended by Peterson *et al.* (9) which contained 5.0% beet molasses, 2.0% malt extract (Difco), 0.75% corn steep liquor, and 0.1%  $(\text{NH}_4)_2\text{HPO}_4$ . It was prepared by adding a sterilized solution of  $(\text{NH}_4)_2\text{HPO}_4$  to the clarified and sterilized solution of molasses, malt extract, and corn steep liquor at the time of inoculation.

Inoculum was grown by transferring the yeast from a stab culture to the sterilized medium and incubating in a reciprocating shaker (84 strokes, 10 cm. length/minute) at 30°C. for 18–24 hours. The culture was centrifuged, washed once with sterile water, and made to the original volume. Five ml. of the suspension, containing about 35–40 mg. of dry yeast, were used as seed for 100 ml. of fermentation medium in a 500 ml. Erlenmeyer flask.

### *Preparation of Fermentation Media*

**Molasses.** Molasses from four different sources were employed in the experiments. They include straight house beet molasses from Michigan Sugar Company, Lansing, Michigan; Steffen beet molasses from American Crystal Sugar Company, Mason City, Iowa, and Great Western Sugar Company, Ovid, Colorado; and Hawaiian black-strap cane molasses from California and Hawaiian Sugar Refining Corporation, Ltd., San Francisco.

**Pretreatment of Molasses.** Clarification of beet molasses could be accomplished by treatment with lime, potassium ferrocyanide or tricalcium phosphate. Lime treatment was used in all the fermentations reported here. Hawaiian cane molasses could not be clarified by any of the above methods, and hence a method involving the use of corn steep liquor was developed. For this, 100 g. of molasses were diluted with 200 ml. of water. To this 20 g. of corn steep liquor was added and the mixture steamed for 20 minutes. After cooling, the pH was adjusted to 8.5 with ammonia and any precipitate was removed by filtration. The filtrate was adjusted to pH 4.6 with sulphuric acid, allowed to stand, filtered, and diluted to the desired concentration.

### *Fermentation*

Fermentation media in most cases contained 0.1%  $\text{KH}_2\text{PO}_4$ , 0.1% urea, 2.0% treated corn steep liquor (obtained by steaming a 1:3 diluted solution of crude corn steep liquor for 30 minutes, cooling, and filtering), and processed molasses sufficient to give approximately 1.0% reducing sugar. After inoculation, the flasks were incubated in the shaker at 30°C., usually for 36 hours. Sugar utilization was 90–95% with *S. cerevisiae*, *T. utilis*, and *C. arborea*, but was somewhat lower (80–87%) with *O. lactis*, due probably to insufficient aeration because of its thick mycelial growth.

## ANALYTICAL METHODS

### *Yeast*

The dry weights of yeasts were determined by centrifuging 10 ml. of homogeneous fermented medium at 2000 r.p.m. for 5 minutes in weighed 6" ×  $\frac{5}{8}$ " Pyrex test tubes,

washing the cells twice with 10 ml. portions of distilled water, drying in an oven at 100°C. for 20 hours, and weighing.

### *Reducing Sugar*

The micro method of Shaffer and Somogyi (17) (reagent No. 50 with 5 g. KI/l.) was used.

### *Protein*

Nitrogen was determined by the Kjeldahl method and protein calculated as  $N \times 6.25$ .

### *Ash*

About 3.0 g. of molasses, after drying, were carbonized in a crucible which was then placed in a muffle furnace at 600°C. for 30 minutes. A carbon-free ash was obtained by adding 5 ml. of 5% ammonium carbonate solution and again heating in the muffle furnace.

### *Phosphorus*

The molasses was treated with  $\text{HNO}_3$ ,  $\text{HClO}_4$ , and HF to get an ash solution in which phosphorus was determined by Fiske and Subbarow's method (18).

### *Vitamins*

Thiamine was determined by the thiochrome method of Conner and Straub (19). The incubation period was reduced to 3-5 hours at 45°C. to avoid long exposure. Experiments involving the addition of pure thiamine chloride hydrochloride yielded recoveries of 85-90% of the added vitamin.

Riboflavin was determined by the microbiological method of Strong and Carpenter (20) and niacin by that of Krehl *et al.* (21). *Lactobacillus casei*, American Type Culture Collection No. 7469, and *Lactobacillus arabinosus*, American Type Culture Collection No. 8014, respectively, were used as test organisms. Folic acid was determined by the microbiological method of Roberts and Snell (22) with *L. casei* as the test organism. Microbiological assays with *Streptococcus faecalis* R, American Type Culture Collection No. 8043, were also run in the earlier stages, but later on were discontinued as the values obtained by both the procedures agreed within an experimental error of  $\pm 8\%$ . In all cases, standards were made up from synthetic crystalline pteroylglutamic acid supplied by Lederle Laboratories, Inc., Pearl River, New York.

## RESULTS

Chemical analysis of the molasses is given in Table I. Lansing beet molasses contained a relatively high amount of nitrogen, while Mason City beet molasses was highest in sugar. Hawaiian cane molasses was low in nitrogen and one of the two highest in ash.

The effect of concentration of sugar upon the rate of yeast growth

TABLE I  
*Chemical Analysis of Molasses Used*

	Lansing beet molasses	Mason City beet molasses	Ovid beet molasses	Hawaiian cane molasses
Sp.gr. (at 20°C.)	1.31	1.46	1.40	1.40
pH (2:1 dilution)	6.7	8.8	8.1	5.4
Moisture, per cent	30.0	31.8	33.8	39.0
Dry matter, per cent	70.0	69.2	66.2	61.0
Total inverted sugar as glucose, per cent	46.5	50.2	43.5	48.4
Total nitrogen, per cent	2.05	1.28	1.23	0.60
Ash, per cent	8.46	10.50	9.30	10.80
Phosphorus, per cent	0.010	0.012	0.005	0.049

is given in Table II. As has been frequently observed with bakers' yeast, higher yields of all yeasts were obtained at a low concentration of sugar.

TABLE II  
*Effect of Concentration of Sugar upon the Rate of Yeast Growth (Hawaiian Molasses)\**

Media	Yeast	8 Hrs.		14 Hrs.		24 Hrs.		36 Hrs.	
		Yield	pH	Yield	pH	Yield	pH	Yield	pH
1.1% sugar +0.1% $\text{KH}_2\text{PO}_4$ +0.1% urea	<i>S. cerevisiae</i>	14.5	4.9	27.8	5.5	36.8	6.4	47.2	6.8
	<i>T. utilis</i>	26.8	4.3	45.5	6.1	49.5	7.0	54.0	7.1
	<i>C. arborea</i>	23.4	4.2	46.5	6.0	56.0	6.8	62.0	6.9
	<i>O. lactis</i>	16.0	5.8	30.0	6.5	58.2	6.9	57.2	7.5
2.2% sugar +0.1% $\text{KH}_2\text{PO}_4$ +0.1% urea	<i>S. cerevisiae</i>	10.0	5.0	15.5	5.5	33.4	6.7	33.4	6.9
	<i>T. utilis</i>	14.9	4.6	27.8	5.6	36.7	6.3	43.5	6.8
	<i>C. arborea</i>	18.6	4.5	39.5	4.8	44.9	5.1	51.3	6.8
	<i>O. lactis</i>	10.0	5.7	24.4	6.1	45.5	6.5	52.4	6.9

\* Yields denote *per cent* dry yeast based on sugar supplied. Initial pH was 4.6 in all cases.

The effect of different amounts of corn steep liquor on the yields of yeast is given in Table III. There was an increase of about 50% in the medium containing 1 ml. of corn steep liquor over that in the media from which it was absent. A further increase resulted from the use of 2 ml. but there was no significant improvement if 4 ml. was used.



TABLE III  
*Effect of Different Amounts of Corn Steep Liquor on the  
 Yield of Yeast (Ovid Beet Molasses)*

Amount of CSL*	Yeasts	Reducing sugar after hydrolysis as glucose	Time	Yield (based as sugar supplied)	pH	
					Initial	Final
<i>ml./100 ml.</i>		<i>g./100 ml.</i>	<i>hrs.</i>	<i>per cent</i>		
None	<i>S. cerevisiae</i>	1.40	36	17.4	5.0	6.5
1 ml.		1.40	36	25.0	5.0	7.0
2 ml.		1.40	36	35.0	5.0	7.1
4 ml.		1.40	36	35.6	5.0	7.2
None	<i>T. utilis</i>	1.40	36	28.5	5.0	6.6
1 ml.		1.40	36	40.2	5.0	7.0
2 ml.		1.40	36	44.0	5.0	7.1
4 ml.		1.40	36	46.0	5.0	7.0
None	<i>C. arborea</i>	1.40	36	39.5	5.0	6.5
1 ml.		1.40	36	44.8	5.0	6.9
2 ml.		1.40	36	62.0	5.0	6.9
4 ml.		1.40	36	59.2	5.0	7.1
None	<i>O. lactis</i>	1.40	36	24.0	5.0	7.0
1 ml.		1.40	36	33.5	5.0	7.2
2 ml.		1.40	36	55.0	5.0	7.5
4 ml.		1.40	36	61.0	5.0	7.8

\* CSL denotes corn steep liquor.

Table IV shows the effect of added nutrients on the yield of yeasts. In evaluating the data, differences of less than 15% are not regarded as being significant (see Table V). In the Lansing molasses the addition of phosphate and urea, individually or combined, had no appreciable effect except in the case of *Candida arborea*, which was benefited by phosphate. Corn steep liquor gave a big improvement for three of the four yeasts, but its combination with phosphate and urea proved to be the best. In the Mason City and Ovid samples, *O. lactis* was the only yeast increased in yield by either urea or phosphate alone. In combination, phosphate and urea gave marked increases in most cases. An improvement in yield, about equal to combined phosphate and urea, was obtained with all yeasts by the addition of corn steep liquor alone. Best yields, however, were obtained with phosphate, urea, and

TABLE IV  
Effect of Added Nutrients on the Yield of Yeasts

Media	Yeasts	Per cent dry yeast after 36 hours, based on sugar supplied			
		Lansing beet molasses	Mason City beet molasses	Ovid beet molasses	Hawaiian cane molasses*
Molasses only	<i>S. cerevisiae</i>	33.5	19.0	18.5	36.6
	<i>T. utilis</i>	41.7	37.0	30.2	39.0
	<i>C. arborea</i>	42.8	40.1	42.5	61.8
	<i>O. lactis</i>	54.1	20.6	23.9	56.5
Molasses +0.1% $\text{KH}_2\text{PO}_4$	<i>S. cerevisiae</i>	35.5	20.6	22.2	36.6
	<i>T. utilis</i>	42.4	33.8	34.4	41.4
	<i>C. arborea</i>	58.3	42.0	45.1	57.0
	<i>O. lactis</i>	59.4	36.2	37.2	56.2
Molasses +0.1% urea	<i>S. cerevisiae</i>	40.6	28.5	23.9	43.5
	<i>T. utilis</i>	41.7	39.8	35.4	48.5
	<i>C. arborea</i>	43.3	45.0	46.0	61.5
	<i>O. lactis</i>	57.2	37.0	36.0	57.8
Molasses +0.1% $\text{KH}_2\text{PO}_4$ +0.1% urea	<i>S. cerevisiae</i>	36.9	35.4	30.2	47.2
	<i>T. utilis</i>	39.1	40.5	39.8	54.0
	<i>C. arborea</i>	58.7	59.0	53.0	62.0
	<i>O. lactis</i>	59.3	57.8	58.5	57.2
Molasses +2 ml. CSL	<i>S. cerevisiae</i>	48.8	38.0	39.8	—
	<i>T. utilis</i>	48.8	44.0	44.2	—
	<i>C. arborea</i>	60.6	59.5	56.0	—
	<i>O. lactis</i>	53.5	52.5	54.7	—
Molasses +0.1% $\text{KH}_2\text{PO}_4$ +0.1% urea +2 ml. CSL	<i>S. cerevisiae</i>	54.3	53.2	44.0	52.7
	<i>T. utilis</i>	65.3	53.5	58.8	62.4
	<i>C. arborea</i>	61.1	60.5	56.1	62.8
	<i>O. lactis</i>	59.8	59.0	60.0	59.2

\* Since the molasses was clarified with a small amount of corn steep liquor, the figures do not apply strictly to the molasses alone or molasses with additions as given in column 1.

corn steep liquor together. Since the Hawaiian molasses had to be clarified with corn steep liquor, no yields could be obtained on molasses alone. No effect was observed with any yeast by adding either phosphate or urea separately, but the two together gave marked increments

TABLE V

*Reproducibility of Yields of Yeasts*(36 hours flask fermentation; figures denote *per cent* dry yeast based on sugar supplied)

Yeast	Lansing beet molasses			Mason City beet molasses			Ovid Colorado beet molasses			Hawaiian cane molasses		
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
<i>S. cerevisiae</i>	50.5	48.3	54.3	—	48.5	53.2	42.7	44.2	44.0	47.0	49.1	52.7
<i>T. utilis</i>	57.0	56.7	65.3	—	57.6	53.5	55.2	58.4	58.8	54.0	59.1	62.4
<i>C. arborea</i>	61.6	63.6	61.1	59.0	61.7	60.5	58.4	55.0	56.1	64.0	60.8	62.8
<i>O. lactis</i>	56.0	59.4	59.8	—	56.1	59.0	60.0	59.2	60.0	—	55.8	59.2

Medium: Molasses equivalent to 1% sugar + 0.1 g.  $\text{KH}_2\text{PO}_4$ , 0.1 g. urea, and 2 ml. treated corn steep liquor/100 ml.

in the yields of *S. cerevisiae* and *T. utilis*. Still higher yields of these two yeasts were obtained by the addition of corn steep liquor to the phosphate-urea combination. In all samples of the molasses, best yields of yeasts were obtained by using 2 ml. of treated corn steep liquor, 0.1 g. urea, and 0.1 g. phosphate/100 ml. of medium. Hence, unless otherwise stated, this was the medium used in all the experiments.

The reproducibility of the yields of yeasts in different runs is given in Table V. Three runs are reported for each molasses and each yeast. In 13 of 16 cases, the variation is within 10% and only in one case does it exceed 12%.

No decisive conclusion can be drawn regarding the superiority of one molasses over the other when the yields are based on the supplied sugar. However, if the amount of molasses required per pound of yeast is calculated, the picture is somewhat different. Such figures for *S. cerevisiae* (Run No. 3) are as follows: Mason City, 3.7 lbs., Hawaiian, 3.9 lbs., Lansing, 4.0 lbs., and Ovid, 5.2 lbs. Hence, for the same amount of yeast, 40% more Ovid than Mason City molasses is required. The differences are somewhat less marked with other yeasts and range from 5.0% for *T. utilis* to 25% for *C. arborea*.

The protein content of the yeasts is given in Table VI. *T. utilis*, *S. cerevisiae*, and *C. arborea* were, in general, equal in protein content, but *O. lactis* was definitely inferior.

The values for the 4 B vitamins in the yeasts are presented in Table

TABLE VI  
Protein ( $N \times 6.25$ ) Content of Yeasts

Yeast	Molasses			
	Lansing beet	Mason City beet	Ovid beet	Hawaiian cane
	per cent	per cent	per cent	per cent
<i>S. cerevisiae</i>	42.5	50.0	53.1	51.8
<i>T. utilis</i>	60.6	43.7	53.7	51.8
<i>C. arborea</i>	47.7	38.8	48.1	49.4
<i>O. lactis</i>	37.5	36.2	31.2	41.9

The media used for the growth of the yeasts contained treated molasses (1% sugar), 0.1%  $KH_2PO_4$ , 0.1% urea, and 2 ml. corn steep liquor. The fermentation was run for 36 hours at 30°C.

VII. Regarding thiamine, riboflavin, and niacin content, *S. cerevisiae*, *T. utilis*, and *C. arborea* gave approximately similar results with all samples of molasses. *O. lactis* was regularly lower in these three vitamins. *T. utilis* and *C. arborea* led the others in riboflavin content, but

TABLE VII  
Vitamin Content of Yeasts ( $\gamma/g.$  of dry yeast)

Molasses	<i>S. cerevisiae</i>				<i>T. utilis</i>				<i>C. arborea</i>				<i>O. lactis</i>			
	Thiamine	Riboflavin	Niacin	Folic acid	Thiamine	Riboflavin	Niacin	Folic acid	Thiamine	Riboflavin	Niacin	Folic acid	Thiamine	Riboflavin	Niacin	Folic acid
Lansing (beet)	37.6	43.8	414.3	21.6	37.5	54.2	520.6	15.2	32.7	69.5	503.1	14.8	20.1	55.0	192.8	7.7
Mason City (beet)	35.7	50.4	443.3	21.4	36.7	62.0	600.0	10.6	33.1	52.3	492.3	16.0	28.9	39.9	212.6	5.6
Ovid (beet)	32.7	45.4	442.8	19.6	33.1	54.8	511.3	11.7	31.3	53.0	512.3	17.6	27.2	42.6	247.5	7.6
Hawaiian (cane blackstrap)	40.8	49.1	568.1	19.1	35.4	58.6	531.4	10.7	33.1	60.0	580.2	15.0	29.0	43.0	242.4	7.8

The media used for the growth of the yeasts was comprised of treated molasses (1% sugar), 0.1%  $KH_2PO_4$ , 0.1% urea, and 2 ml. corn steep liquor. The fermentation was run for 36 hours at 30°C.

*O. lactis* unexpectedly was found to contain as much riboflavin as *S. cerevisiae*. As regards folic acid, *S. cerevisiae* proved to be the best source, followed in order by *C. arborea*, *T. utilis*, and *O. lactis*. The last named was particularly low in folic acid.

In Table VIII we have compared the data on the yield and B vitamin content of *Torula utilis* with that reported by other workers using different substrates. Molasses has given the highest yields of this yeast. Our yields, ranging from 53 to 65%, check very well with those reported by Lewis *et al.* (14). Regarding vitamins, our niacin values run almost parallel to those reported by Lewis *et al.* (14) and riboflavin to those of Thaysen and associates (12). Figures for folic acid are given only by Kurth and Cheldelin (11) on wood sugar stillage and are much lower than our values on molasses.

TABLE VIII  
*Comparison of Yield and Vitamin Content of Torula utilis*  
*Grown on Different Sugar Substrates*

Sugar substrate	Yield of dry yeast (based on sugar supplied)	Vitamin content/g. dry yeast				Reference
		Thiamine	Riboflavin	Niacin	Folic acid	
	<i>per cent</i>					
Wood sugar stillage	48	6.2	49	500	2.8	(11)
Fruit juice	53	22	44	535	—	(14)
Molasses						
1. Thaysen and associates	—	22	54	440-470	—	(12)
2. Lewis <i>et al.</i>	56	18	36	610	—	(14)
3. Authors*	58-65	35-38	55-62	511-600	10-15	

\* The figures denote the range of yields and vitamins on four different molasses.

#### ACKNOWLEDGMENT

The authors are indebted to Professor Elizabeth McCoy of the Department of Agricultural Bacteriology for transplants of the yeast cultures and to Professor M. J. Johnson, University of Wisconsin, for helpful suggestions. Two of us (P. N. Agarwal and K. Singh) are indebted to the Government of India for scholarships for graduate study in the United States. Another of us (P. S. King) is indebted to the National Bureau of Industrial Research of the Ministry of Economic Affairs, China, for financial aid during the period March, 1945 to August, 1946.

#### SUMMARY

*Saccharomyces cerevisiae*, *Torula utilis*, *Candida arborea*, and *Oidium lactis* were grown on 4 kinds of cane and beet molasses. Yields of these yeasts ranging from 44 to 65% based on sugar supplied were obtained.

Approximately 90% of the sugar supplied was fermented. The addition of nutrients such as phosphate, urea, and corn steep liquor to the molasses was necessary to obtain the highest yields.

The protein content of these yeasts ranged from 31 to 69%.

The yeasts were assayed for their content of thiamine, riboflavin, niacin, and folic acid. The values ranged from 20–40  $\gamma$  for thiamine, 40–69  $\gamma$  for riboflavin, 192–600  $\gamma$  for niacin, and 6–22  $\gamma$  for folic acid/g. of dry yeast.

As a rich source of protein and B vitamins, the 4 yeasts fall in the following order: *T. utilis*, *C. arborea*, and *S. cerevisiae* about equally good, and *O. lactis* distinctly poorer than the other three.

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# Esters of DL-Lactic Acid with Glycerol and Fructose<sup>1</sup>

Leonhard Feldmann and Hermann O. L. Fischer

*From the Department of Chemistry, Banting Institute, University of Toronto*

Received February 20, 1947

In 1913, in a comprehensive review on the "Synthesis of Depsides, Lichen Compounds and Tannins," Emil Fischer (1) called attention to the possible occurrence of esters of  $\alpha$ -oxyacids with glycerol and sugar in the plant kingdom. He suggested that esters of these acids, notably glycolic acid, with sugar might be found in sweet fruits. These fruits in the unripe state contain considerable amounts of free glycolic or related acids. The ripening process might involve removal of the free acids through esterification with sugar.

From the beginning it was apparent that the very nature of esters of glycolic or lactic acid with polyalcohols would make their isolation from natural material difficult. Therefore, their chemical synthesis, prior to any attempt at isolation, seemed advisable in order to determine their solubilities, rates of hydrolysis, and other properties.

In the case of glycolic acid, the ester synthesis was carried out by Bruno Gohlke (2) and one of the present authors, as follows:

Monochloroacetic acid was benzylated by means of sodium benzyolate in benzylalcohol and the free O-benzylglycolic acid was transformed into its acid chloride by treatment with phosphorus pentachloride. O-benzylglycolic acid chloride was combined, in the presence of tertiary bases, with glycolic acid, acetone glycerol, glycerol,  $\beta$ -diacetonefructose, monoacetoneglucose and glucose. In every instance, the coupling and subsequent catalytic removal of the benzyl residue could be readily achieved in good yield.

Analogous to the aforementioned paper on esters of glycolic acid, the esters of DL-lactic acid were prepared via  $\alpha$ -bromopropionic acid, O-benzyl-DL-lactic acid and its chloride, followed by the usual esterification with polyalcohols in the presence of tertiary bases and the subsequent catalytic removal of the protective benzyl groups. In the experimental part of this paper we describe the preparation of  $\alpha$ -lactyl-

<sup>1</sup> Dedicated to Professor Carl Neuberg on his 70th birthday.

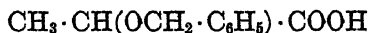


glycerol, trilactylglycerol and monolactyl- $\beta$ -diacetonefructose. The two glycerol esters were characterized by their acetyl derivatives, since attempts to distil them or obtain them in crystalline form had not been successful. An investigation with this synthetic material to determine whether triglycolyl- or trilactylglycerols can be hydrolyzed by lipases under physiological conditions should be worth while. Such an investigation would involve difficulties, since these glycerides are hydrolyzed even by cold aqueous sodium bicarbonate solution. Should this type of "water-soluble fats" occur in nature, isolation would thus be possible only under special precautions.

If the interest in the lactic acid esters which we have prepared should be great enough to justify the rather burdensome preparation of derivatives of the natural lactic acid, it would be necessary to start from the D-lactic acid. In this case one could consider utilizing the carbobenzoxy method of Bergmann and Zervas (3). An incentive for this synthesis lies in the supposition that formation of lactic acid esters with glycerol, or sugar, may be utilized in animal organisms for the purpose of quickly neutralizing lactic acid, which occurs in the muscle as a breakdown product of glucose and as an intermediary step toward subsequent resynthesis into carbohydrates.

## EXPERIMENTAL

### *O-Benzylactic Acid*



Sodium metal (16.5 g.; 2.1 mols.) in pea-sized lumps were gradually added to 500 cc. of purified benzyl alcohol. The metal dissolved slowly with evolution of hydrogen and considerable heat. Into the still warm solution (temp. ca. 130°C.) 50 g. of  $\alpha$ -bromopropionic acid (Kahlbaum) were poured in a thin stream, with shaking. The reaction mixture was kept over night at room temperature, the sodium bromide formed was removed by centrifuging and the solution was concentrated under reduced pressure at a bath temperature of 120°C. After dissolving the residue in 200 cc. of water, the solution was twice extracted with 100 cc. of ether to remove traces of benzyl alcohol. After acidification of the now clear aqueous solution with 30 cc. of concentrated  $\text{H}_2\text{SO}_4$ , the free benzylactic acid separated as an oil and was extracted with ether. The ethereal solution was dried with anhydrous sodium sulfate and fractionated after evaporation of the ether. After the small forerun, a water-clear oil distilled at 130°C., under 0.1 mm. pressure; b.p. 117°C. at 0.05 mm. pressure, after a second distillation. The pure substance crystallized soon after rubbing. Yield 43.6 g.; 74% of theory. For analysis, the substance was redistilled and recrystallized from ether-petroleum ether, at -20°C. It then melted at 41-42°C. after sintering.

$C_{10}H_{12}O_3$  (180) Calc. : C 66.7; H 6.7%.  
Found: C 66.96; H 7.04%.

The benzylactic acid is readily soluble in ether, alcohol, and chloroform, but is almost insoluble in water and petroleum ether.

*O-Benzylactic Acid Chloride,*



Twenty g. of *O*-benzylactic acid were mixed with 20 g. of thionyl chloride and refluxed for 15 minutes on the water bath under anhydrous conditions. The excess of thionyl chloride was distilled off under reduced pressure at a bath temperature of 40–45°C.; after discarding a slight forerun at 100–105°C., using a high vacuum pump, the residue distilled at 0.12 mm. pressure. B.p. of the colorless oil 91°C. at 0.05 mm.; 124°C. at 14 mm. Yield of a twice-distilled product 18.5 g.; 84% of theory.

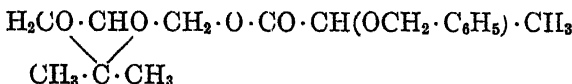
$C_{10}H_{11}O_2Cl$  (198.5) Calc. : C 60.45; H 5.59; Cl 17.86%  
Found: C 60.08; H 5.55; Cl 18.77%

*Anilide*

*O*-benzylactic acid chloride (0.82 g. in 3 cc. of dry chloroform) were mixed with 1 g. of aniline in 22 cc. of absolute ether. The solution was treated with water, dilute  $H_2SO_4$  and  $NaHCO_3$  solution, and finally washed with water. After evaporation of the ether at reduced pressure, the residue crystallized. The *O*-benzylactic acid anilide was recrystallized twice from alcohol-water, and dried *in vacuo* at 56°C., m.p. 74°C.

$C_{16}H_{17}O_2N$  (255) Calc. : N 5.5%  
Found: N 5.7%

*α-(Benzylactyl)acetoneglycerol,*

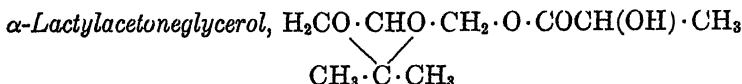


To a solution of 15 g. of *O*-benzylactic acid chloride in 20 cc. of dry chloroform, 12.5 cc. of dry pyridine were added drop by drop, with cooling in a freezing mixture. Then 10 g. of acetoneglycerol were added. After a short while, pyridine hydrochloride crystallized from the slightly yellow solution. The reaction mixture was kept at room temperature for two days, diluted with ether and water, treated with dilute  $H_2SO_4$ ,  $NaHCO_3$  solution, and finally washed with water. The ethereal solution was dried with anhydrous sodium sulfate, the ether was evaporated under reduced pressure, and the residue distilled at 150°C. at 0.2 mm. After being twice distilled at 150°C. under 0.2 mm. pressure, the  $\alpha$ -(benzylactyl)acetoneglycerol was analytically pure. Yield: 15.6 g.; 70.2% of theory.

$$n_D^{16^\circ} = 1.4916.$$

$$\text{C}_{16}\text{H}_{22}\text{O}_5 \text{ (294)} \quad \text{Calc. : C 65.3; H 7.5\%}$$

$$\text{Found: C 65.39; H 7.6\%}$$



Catalytic hydrogenation was used to split out the benzyl residue from the  $\alpha$ -(benzyl-lactyl)acetoneglycerol:

0.62 g. of palladium catalyst (4) were saturated with hydrogen while suspended in 20 cc. of glacial acetic acid (purified by boiling with chromic acid) in a shaking vessel. Excluding air, a solution of 1.5 g. of  $\alpha$ -(benzyl-lactyl)acetoneglycerol in 50 cc. of glacial acetic acid was added. In 25 minutes, 134.2 cc. of hydrogen (at 25.5°C. and 753 mm.) were absorbed under shaking (calc. 126 cc.). The catalyst was filtered off, the acetic acid evaporated *in vacuo* at a bath temperature of 45°C. and the residue distilled at 0.2 mm. at a bath temperature of 110°C. Yield: 0.7 g.; 67.3% of theory. For analysis, the water-clear oil was redistilled. B.p. 92°C., under 0.3 mm. pressure.

$$n_D^{22^\circ} = 1.4438.$$

$$\text{C}_9\text{H}_{16}\text{O}_5 \text{ (204)} \quad \text{Calc. : C 52.9; H 7.8\%}$$

$$\text{Found: C 52.78; H 7.95\%}$$

*$\alpha$ -Lactylglycerol,*

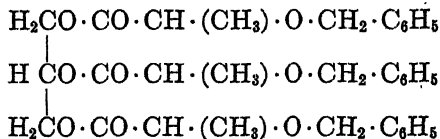


$\alpha$ -Lactylacetoneglycerol (0.9 g.) were heated on the water bath for one hour with 5 cc. of 12.5% acetic acid. The solution was concentrated, under reduced pressure, at a bath temperature of 45°C. and the residue dissolved in water and re-concentrated several times under the same conditions, to remove the acetic acid completely. Finally, the solution is evaporated to dryness at a bath temperature of 60°C. It remained a water-soluble, non-distillable oil, which was not further purified. For identification purposes it was transformed into the 1-(acetyl-lactyl)-2,3-diacetyl-glycerol by treating it, under cooling, with 5 cc. of a mixture of equal parts of pyridine and acetic anhydride. After standing two days at room temperature, excess pyridine and acetic anhydride were distilled off under reduced pressure, at a bath temperature of 45°C. The solution of the residue in chloroform was washed with dilute  $\text{H}_2\text{SO}_4$ ,  $\text{KHCO}_3$  solution and water, and finally dried by means of anhydrous  $\text{Na}_2\text{SO}_4$ . After evaporation of the chloroform at reduced pressure, the substance distilled at 0.3 mm. pressure at a bath temperature of 150–155°C. After two distillations the substance was analytically pure.

$$n_D^{21.5^\circ} = 1.4377.$$

$$\text{C}_{12}\text{H}_{18}\text{O}_8 \text{ (290)} \quad \text{Calc. : C 49.65; H 6.25\%}$$

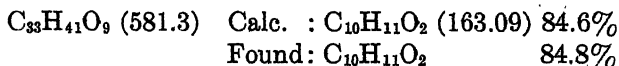
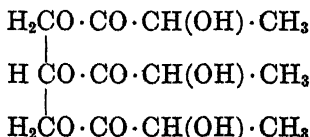
$$\text{Found: C 49.80; H 6.29\%}$$

*Tribenzylactylglycerol,*

Dry glycerol (4.2 g. dissolved in 20 cc. of dry chloroform) was mixed under careful cooling with 27.5 g. (3.3 mols.) O-benzylactic acid chloride. To this solution 15 cc. of dry pyridine in 15 cc. of dry chloroform were slowly added with shaking, the solution turning slightly yellow. After standing from two to three days at room temperature, the reaction mixture was diluted with ether, then treated with water, dilute  $\text{H}_2\text{SO}_4$ ,  $\text{NaHCO}_3$  solution and finally washed again with water. The ethereal solution was dried with anhydrous  $\text{Na}_2\text{SO}_4$ , and the ether evaporated under reduced pressure. The residue was a slightly yellowish oil, decomposing upon attempting to distil it in a vacuum of 0.5 mm. Therefore, to purify the oil, it was twice dissolved in ethyl acetate, and reprecipitated with petroleum ether. The solvents were decanted and the residue taken up in a small volume of ethyl acetate, or methanol, and slowly concentrated in an open dish in the vacuum desiccator. Finally, it was kept for several hours in a high vacuum over phosphorus pentoxide. Yield 19.7 g.; 74% of theory.

*Titration.* To 0.0956 g. of substance in 10 cc. of methanol, 2 cc. of  $N$  NaOH solution were added. After  $1\frac{1}{4}$  hours it was back titrated with 0.1  $N$   $\text{H}_2\text{SO}_4$ ; indicator: phenolphthalein.

Consumed 4.97 cc. of 0.1  $N$  NaOH solution.

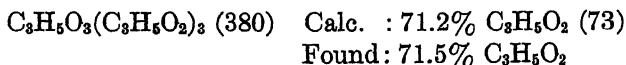
*Trilactylglycerol,*

Palladium catalyst (0.63 g. suspended in 10 cc. of glacial acetic acid) was saturated with hydrogen, and to this 7.25 g. of tri(benzylactyl)glycerol in 50 cc. of glacial acetic acid were added. The mixture was shaken with hydrogen until reduction was complete. In one hour and twenty-five minutes, at  $18.5^\circ\text{C}$ . and 754 mm. pressure, 904 cc. of hydrogen (calc. 902 cc.) were taken up. The catalyst was filtered off, the glacial acetic acid evaporated under reduced pressure, at a bath temperature of  $40^\circ\text{C}$ . and the residue kept under vacuum for one hour longer. The residue was dissolved twice in methanol and the methanol evaporated *in vacuo* at a bath temperature of  $30^\circ\text{C}$ . Finally, the substance was kept for  $2\frac{1}{2}$  hours at 0.4 mm. pressure and  $40^\circ\text{C}$ . The residue was a syrup, which was taken up in a small amount of methanol. The methanol was removed by standing in an open dish, then in a vacuum desiccator

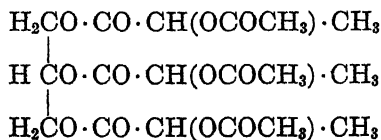
over  $P_2O_5$ , and finally in the high vacuum with frequent agitation of the substance. Yield 3.55 g.; 94% of theory. Easily soluble in water, methanol and ethanol.

*Titration.* 0.1385 g. substance were dissolved in 10 cc. of methanol, 25 cc. of 0.1  $N$   $NaOH$  were added and back titrated after  $1\frac{1}{2}$  hours with 0.1  $N$   $H_2SO_4$ .

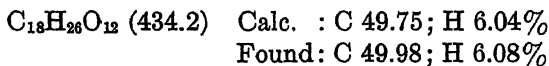
Consumed 13.55 cc. of 0.1  $N$   $NaOH$ .



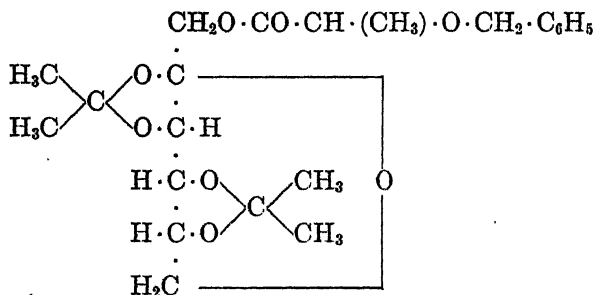
*Triacetylactylglycerol,*



A mixture of 5 cc. of acetic anhydride and 5 cc. of dry pyridine was poured over 1 g. of trilactylglycerol, and the mixture shaken, with cooling, until the latter was dissolved. After standing at room temperature for 24 hours, the excess of anhydride and pyridine was evaporated under reduced pressure and the residue taken up in chloroform. The chloroform solution was washed successively with water, dilute  $H_2SO_4$ ,  $NaHCO_3$  solution, and again with water, and finally dried with anhydrous  $Na_2SO_4$ . The chloroform was evaporated under reduced pressure, at  $35^\circ C$ ., and the residue distilled at 0.2 mm. at a bath temperature of  $215-220^\circ C$ . After a second distillation at 0.2 mm. at a bath temperature of  $205-210^\circ C$ ., the product, an extremely viscous oil, was analytically pure.



*Monobenzylactyl- $\beta$ -diacetonefructose,*

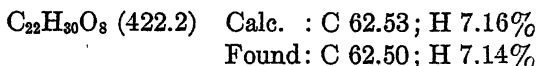


Ten g. (ca. 1.1 mol.) of benzylactic acid chloride were dissolved in 35 cc. of dry chloroform, and to the solution, while shaking and cooling with a freezing mixture, 10 g. of  $\beta$ -diacetonefructose (5) were added. The sugar derivative dissolved rapidly. Then 7.5 cc. of dry pyridine in 15 cc. of dry chloroform were slowly added, drop by

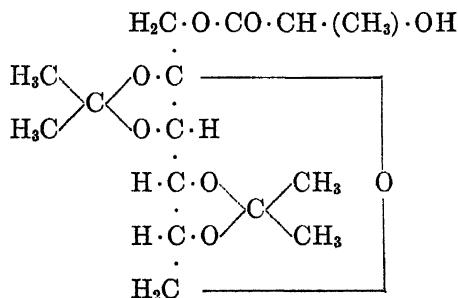
drop. After being kept for 45 hours at 37°C. the solution became slightly reddish. It was now diluted with the same volume of chloroform, washed with ice-cold water, then with dilute  $\text{H}_2\text{SO}_4$  and then with water again. The chloroform solution was filtered through a dry filter paper, dried with anhydrous  $\text{Na}_2\text{SO}_4$  and the remaining reddish syrup kept in an open dish in the vacuum desiccator over  $\text{P}_2\text{O}_5$ . After three days, needle-like crystals formed in the syrup. The mixture of crystals were spread on a porous plate and a colorless crystalline substance resulted. Yield: 3.55 g.; 22% of theory.

These same crystals could also be obtained by crystallization from alcohol-water.

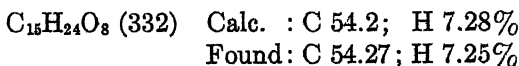
For analysis the preparation was recrystallized from a mixture of alcohol and water and dried in a high vacuum over  $\text{P}_2\text{O}_5$ . Heated in the capillary, it melted at 65–68°C.; sintered at 65°C. and was completely molten at 68°C.



*Monolactyl-β-diacetonefructose,*



One g. monobenzylactyl-β-diacetonefructose in 30 cc. of glacial acetic acid were added to 0.5 g. of palladium catalyst in 10 cc. of acetic acid, the catalyst having been previously saturated with hydrogen. In less than 10 minutes the absorption of hydrogen was complete; 66 cc. at 22°C. and 756 mm. (calc. 57.6 cc.). The catalyst was filtered off and the solution concentrated at a bath temperature of 40°C. under reduced pressure. To remove the acetic acid completely, high vacuum was applied. The residue, a colorless oil, distilled at 0.5 mm. and 170–180°C. Yield: 0.5 g.; 66% of theory.



### SUMMARY

The chemical synthesis of α-lactylglycerol, trilactylglycerol and monolactyl-β-diacetone-D-fructose is described. The possible biological significance of this type of α-oxyacid ester is discussed.

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# Preparation of 3-Hydroxy- $\Delta^{9,11}$ -12-Ketocholenic Acid and its Lower Homologues <sup>1</sup>

Erwin Schwenk and Elsie Stahl

*Chemical Research Laboratories of Schering Corporation, Bloomfield, N. J.*

Received February 20, 1947

## INTRODUCTION

One of the important intermediates for the preparation of steroids substituted in position 11 by oxygen is 3-hydroxy- $\Delta^{9,11}$ -12-ketocholenic acid. This substance was first prepared by Chakravorty and Wallis (1) by the bromination of 3-acetoxy-12-ketocholanic acid followed by the elimination of hydrogen bromide upon treatment with ethoxide in alcohol.

It was thought that it should be possible to introduce oxygen into the 11-position of 3-acetoxy-12-ketocholanic acid by treatment of this acid with selenium dioxide. Surprisingly, however, it was found that the reaction takes a different course in the cholanic acid series. The product which was obtained when 3-acetoxy-12-ketocholanic acid was submitted to the action of selenious acid in acetic acid solution was identical with the compound previously obtained by Chakravorty and Wallis (1). Instead of oxygen being introduced into the molecule, dehydrogenation had taken place by the elimination of one atom of hydrogen from each of adjacent C-9 and C-11 positions.

Only a few instances have been reported where the treatment of organic substances with selenium dioxide or selenious acid resulted in the elimination of two hydrogen atoms from adjacent methylene groups (4, 5). This mode of reaction seems to occur more easily in the steroids than in other classes of compounds. Thus, Callow and Rosenheim (6) in the ergosterol series and Callow (7) with apocholic acid reported the elimination of hydrogen without introduction of oxygen.

The new reaction is not restricted to the 3-acetoxy-12-ketocholanic acid. It was found that the corresponding nor-, bismor-, and etiocholanic acids were also dehydrogenated to the  $\Delta^{9,11}$  compounds. Furthermore,

<sup>1</sup> Dedicated to Professor Carl Neuberg on his 70th birthday.



it was found that not only the 3-acetoxy, but also the easily obtainable 3-succinoxy derivatives of these acids (8) can be used in the reaction with selenious acid.

The presence of the double bond in the substances obtained was shown both by the analysis and by the ultraviolet absorption spectrum, where all of the substances showed an absorption maximum at  $240\text{ m}\mu$ .<sup>2</sup>

### EXPERIMENTAL

#### $\Delta^9, 11$ -3-Hydroxy-12-ketocholenic Acid

A solution of 33.4 g. of 3-acetoxy-12-ketocholenic acid (m.p.  $198.5\text{--}200.5^\circ\text{C}$ .) in 150 cc. of acetic acid and 16.7 g. of selenious acid (240% excess) was refluxed for 8 hours. The reaction mixture was then filtered from the precipitated selenium through a sintered glass funnel and the filtrate poured into ice water. After filtration, a 3 g. portion of the product was saponified and the sodium salt of the acid was precipitated by the addition of solid sodium chloride. The sodium salt was redissolved in water and the solution acidified. After crystallization of the precipitated acid from dilute methanol and from ethyl acetate the m.p. was  $173.5\text{--}174.5^\circ$  (literature  $172\text{--}173^\circ$ , Chakravorty and Wallis (1);  $173\text{--}174^\circ$ , Seebeck and Reichstein (9)).

#### 3-Succinoxy- $\Delta^9, 11$ -12-ketocholenic Acid

A solution of 4.9 g. of 3-succinoxy-12-ketocholenic acid, 30 cc. of acetic acid and 2 g. of selenious acid (210% excess) was refluxed for 5 hours and then filtered free from selenium. The filtrate was poured into ice water and the product was crystallized from dilute acetone using Darco. Two fractions were obtained: I, 3.7 g., m.p.  $231.8\text{--}234.5^\circ\text{C}$ . and II, 1.0 g., m.p.  $198.5\text{--}203.5^\circ\text{C}$ .

A small amount of fraction I was recrystallized from ethyl acetate, m.p.  $233.5\text{--}235.5^\circ$ . The analysis showed it to be 3-succinoxy- $\Delta^9, 11$ -12-ketocholenic acid.

Anal: Calc'd for  $\text{C}_{23}\text{H}_{40}\text{O}_7$ : C, 68.83; H, 8.25

Found: C, 69.02; H, 8.36

The remaining material was saponified and 2.0 g. of crude 3-hydroxy- $\Delta^9, 11$ -12-ketocholenic acid was obtained, which, after recrystallization from ethyl acetate, gave 1.3 g. of the pure acid, m.p.  $171.5^\circ$ ,  $172.5\text{--}173.5^\circ$ . Esterification of 1.0 g. using the method of Freudenberg and Jakob (10) was effected by dissolving in 10 cc. methanol and adding two drops of acetyl chloride. After standing overnight, the solution was processed in the usual manner. No acid was reclaimed. The ester was recrystallized from ether-petroleum ether and then from ethyl acetate-petroleum ether, m.p.  $115\text{--}118^\circ$ , (literature  $115\text{--}116^\circ$ , Seebeck and Reichstein (9)).

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<sup>2</sup> Dr. E. C. Kendall and his coworkers have studied our reaction thoroughly in connection with their work on the synthesis of dehydrocorticosterone and have established the most advantageous conditions for yield, purity and the elimination of traces of selenium from the final product. They will report on their work separately.

*3-Hydroxy- $\Delta^{9,11}$ -12-ketonorcholenic Acid*

A solution of 3.1 g. of 3-acetoxy-12-ketonorcholenic acid (Schwenk *et al.*) (8) in 12 cc. of acetic acid and 1.6 g. of selenious acid (233% excess) was refluxed for 5 hours. The reaction mixture was diluted with methanol and filtered from the precipitated selenium, after which the filtrate was poured into ice and water and the precipitate collected on a filter. The insoluble compound was saponified with aqueous alkali and the acid recrystallized from dilute acetone. The first crystallizate weighed 2 g., m.p. 253–254.5°C. Analysis showed it to be 3-hydroxy- $\Delta^{9,11}$ -12-ketonorcholenic acid,  $[\alpha]_D^{20} = +92.7^\circ$  (dioxane).

Anal.: Calc'd for  $C_{23}H_{34}O_4$ : C, 73.76; H, 9.15  
Found: C, 73.46; H, 9.37

*3-Acetoxy- $\Delta^{9,11}$ -12-ketonorcholenic Acid*

The 3-hydroxy- $\Delta^{9,11}$ -12-ketonorcholenic acid was acetylated with pyridine and acetic anhydride and recrystallized from dilute methanol, m.p. 192–194°C.

Anal.: Calc'd for  $C_{25}H_{36}O_5$ : C, 72.08; H, 8.71  
Found: C, 71.98; H, 8.50

*Methyl 3-Hydroxy- $\Delta^{9,11}$ -12-ketonorcholenate*

One g. of 3-hydroxy- $\Delta^{9,11}$ -12-ketonorcholenic acid was dissolved in 10 cc. methanol and .04 cc. acetyl chloride added carefully. After standing overnight, the solution was poured into water and the solid separated by filtration. No acid could be reclaimed and the crude ester was crystallized from ether-petroleum ether. The main fraction consisted of 0.8 g., m.p. 141–143°C. A small amount was recrystallized from dilute acetone, m.p. 139–140°C.;  $[\alpha]_D^{20} = +89.7^\circ$  (dioxane).

Anal.: Calc'd for  $C_{24}H_{36}O_4$ : C, 74.18; H, 9.34  
Found: C, 75.82; H, 9.54

*Methyl 3-Acetoxy- $\Delta^{9,11}$ -12-ketonorcholenate*

One-half g. of methyl 3-hydroxy- $\Delta^{9,11}$ -12-ketocholenate was acetylated in pyridine with acetic anhydride. The product was recrystallized from dilute methanol, m.p. 190–192°C.,  $[\alpha]_D^{20} = +99.7^\circ$  (dioxane); (literature 197–199°C.,  $[\alpha]_D^{20} = +94.6^\circ \pm 3^\circ$  (acetone), Lardon and Reichstein (11)).

*3-Hydroxy- $\Delta^{9,11}$ -12-ketobisnorcholenic Acid*

To a solution of 1.6 g. of 3-acetoxy-12-ketobisnorcholenic acid (Schwenk *et al.*) (8) in 8 cc. of acetic acid was added 0.9 g. of selenious acid (249% excess) and the mixture was refluxed 5 hours. After dilution with methanol it was filtered from the precipitated selenium. The filtrate was poured into ice water and the precipitate collected on a filter. The compound was then saponified with aqueous alkali and the

acid thus obtained was recrystallized from dilute acetone. There was obtained 0.4 g. of material, m.p. 294–296°C.,  $[\alpha]_D^{20} = +79.7^\circ$  (dioxane).

Anal.: Calc'd for  $C_{22}H_{32}O_4$ : C, 73.30; H, 8.94  
Found: C, 73.07; H, 9.24

### *3-Acetoxy- $\Delta^9,11$ -12-ketobisnorcholenic Acid*

In another experiment 1 g. of 3-acetoxy-12-ketobisnorcholenic acid was refluxed with 8 cc. of acetic acid and 0.48 of selenious acid (200% excess) for 1½ hours. The mixture was diluted with methanol and filtered from precipitated selenium. The filtrate was poured in ice water containing a small amount of hydrochloric acid. The precipitate was recrystallized without previous saponification from dilute acetone with Darco and gave 0.65 g. of material melting at 249°C.

Anal.: Calc'd for  $C_{24}H_{34}O_5$ : C, 71.61; H, 8.51  
Found: C, 71.72; H, 8.81

The same substance was obtained from the acetylation of 3-hydroxy- $\Delta^9,11$ -12-ketobisnorcholenic acid with pyridine and acetic anhydride.

### *Methyl 3-Hydroxy- $\Delta^9,11$ -12-ketobisnorcholenate*

To a solution of 150 mg. of 3-hydroxy- $\Delta^9,11$ -12-ketobis(norcholenic) acid in 10 cc. of methanol was added four drops of acetyl chloride. After standing for one day the mixture was poured into ice water, freed from unreacted acid by extraction of the ether solution with bicarbonate solution and recrystallized from ether-petroleum ether, m.p. 152–153°C.

Anal.: Calc'd for  $C_{23}H_{34}O_4$ : C, 73.76; H, 9.15  
Found: C, 73.62; H, 9.40

### *3-Acetoxy- $\Delta^9,11$ -12-ketoetiocholenic Acid*

A 0.700 g. portion of 3-acetoxy-12-ketoetiocholenic acid (Schwenk *et al.*) (8) was refluxed for 10 hours with 0.34 g. of selenious acid (183% excess) and 5 cc. of acetic acid. After dilution with methanol, the selenium was separated by filtration and the solution was poured into ice water. The cream-colored precipitate was filtered and dried. The yield was 0.62 g. This crude product was recrystallized once from dilute acetone using Darco and a second time from dilute acetone alone. There was obtained 0.43 g. of slightly yellowish needles, m.p. 214.3–214.8°C.

Anal.: Calc'd for  $C_{23}H_{30}O_5$ : C, 70.56; H, 8.08  
Found: C, 70.89; H, 8.26

Another similar experiment gave the same substance, m.p. 210.3–212°C.,  $[\alpha]_D = +142^\circ$  (dioxane).

Anal.: Calc'd for  $C_{23}H_{30}O_5$ : C, 70.56; H, 8.08  
Found: C, 70.41; H, 8.26

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# Notes on the Purification and Determination of Codehydrogenase I (Cozymase)<sup>1</sup>

F. Schlenk and T. Schlenk

*The University of Texas: Medical Branch, Galveston, and The M. D. Anderson  
Hospital for Cancer Research, Houston*

Received February 24, 1947

## INTRODUCTION

For studies in the field of carbohydrate metabolism the availability of pure enzymes, coenzymes, and intermediates has proved to be of decisive importance. Unfortunately, the preparation of these compounds is attended with considerable expenditure of time and equipment. It is not surprising, therefore, that attempts have been numerous to simplify the preparative procedures. Thanks particularly to the investigations of C. Neuberg and his coworkers (1), some of the more important carbohydrate phosphoric acid esters are now easily available in quantity. The preparation of coenzymes is longer and much more difficult, especially codehydrogenase I (cozymase, diphosphopyridine nucleotide). Methods for its preparation, together with rigorous proof of the purity attained, have been reported from the institutes of von Euler (2), Meyerhof (3), Warburg (4), and by the present authors (5). The laboriousness of these methods has been deplored sometimes, and simplified procedures have been communicated. Some of them aim at securing crude preparations only and consist essentially of a combination of previously known steps. A few valuable new features have been introduced, however. Unfortunately, the stamina of some investigators seems to have given way too soon in furnishing proof for their claims for purity. It is the purpose of this paper to offer some suggestions pertaining to purification and test procedures.

## PREPARATION OF COZYMASE

### *Source Material and Procedures*

Brewer's or baker's yeast is most commonly used and several procedures have been described (2, 3, 5) which will give preparations of

<sup>1</sup> Dedicated to Professor Carl Neuberg on his 70th birthday.

high purity. The yield from erythrocytes (4) is too low to make the isolation practical. Where yeast is not available, the method of Ochoa (6) using muscle may be resorted to.

New procedures have been reported by Jandorf (7) and by Sumner and coworkers (8). Both methods have been repeated in this laboratory with good results.<sup>2</sup> In the isolation procedure of Jandorf we prefer to use water instead of 0.5 *N* HCl for extraction, because it leaves intact the dihydrocozymase which is sensitive to acid. In the extraction with hot water dihydrocozymase is oxidized to cozymase. We also recommend that the temperature during vacuum distillations be kept at 40–45°C. The final product contains adenylic acid and related compounds which can be removed by alcoholic fractionation of the lead salts (5). We also found the alcohol and ether extraction proposed by Sumner, Krishnan, and Sisler (8) very excellent. A combination of these two methods with some of the previously employed steps should enhance the general availability of pure cozymase.

#### *Impurities in Cozymase Preparations*

The most tenacious impurities of cozymase are adenosine-5'-mono- and diphosphate, and the compound consisting of adenine-ribose-pyrophosphate-ribose. These products not only follow cozymase through many of the purification steps, but they are also formed readily from cozymase by the action of alkali (9), or by prolonged storage of the coenzyme in solution (after some weeks) or in the dry state (after some months). This decomposition has been responsible for many errors in the past. Cozymase has been given a role in the phosphorylation processes, which may have been due to these decomposition products. Then again, excessively high values for cozymase were found in tissues when partially decomposed cozymase was used as a standard. In some instances the purity of cozymase obtained by new procedures has been overrated simply because it was compared with a preparation obtained from another institute.

It should be emphasized that cozymase in contact with crude enzyme preparations or tissue homogenates is always a potential source of adenylic acid. The use of pure cozymase for determination of its specificity as coenzyme is sensible only if pure proteins are available.

<sup>2</sup> The authors are indebted to Dr. G. S. Bratton, Anheuser-Busch, Inc., St. Louis, for a generous supply of yeast.

*Determination of the Purity of Cozymase Preparations*

There are a large number of test procedures which may be classified in two groups: biochemical tests in which the catalytic power as coenzyme is measured; and chemical or physical examination. The more important tests will be listed here and some improvements as well as possible pitfalls will be reported.

## BIOCHEMICAL TEST REACTIONS

*Fermentation Test with Apozymase.* The term apozymase has been coined by C. Neuberg (11) to designate yeast preparations from which cozymase has been removed. The fermenting power can be restored by the addition of cozymase. The apozymase test originally elaborated for a special fermentation apparatus (12, 13) has been adapted by Schlenk and Vowles (14) for use in the more generally available Warburg apparatus. It has been used and improved by other investigators (15, 16). Some simplifications will be described here.

Apozymase is prepared from dried brewer's yeast by washing at 15–20°C. Two gs. are suspended in 100 ml. of cooled distilled water in a centrifuge tube. Stopper and shake well until a homogenous suspension is obtained. Begin to centrifuge after 7 minutes. A maximum speed of 2,000 r.p.m. should be reached, and the centrifugation should be finished after 7–10 minutes. Repeat this washing process. A total of 3 or 4 washings, taking about an hour, will be found sufficient. Prolonged washing will lower the blank CO<sub>2</sub> output in the manometric experiment, but will also cause less activation by added cozymase. A few trials for each batch of dried yeast will reveal the optimum conditions. Dried yeast, if stored cold and protected from moisture, will retain its apozymase potentiality for at least a year. For the experiment, suspend the apozymase in 7.0 ml. of 20% glucose solution, 7.0 ml. of 0.5 M phosphate buffer, pH 6.2, and 2.0 ml. of 0.1 M Mg SO<sub>4</sub> solution. Transfer 1.0 ml. of this mixture into the main compartment of each Warburg flask (any type), shaking carefully between pipettings to insure uniformity of the suspension. Add 0.25 ml. of a sodium hexosediphosphate solution containing approximately 10 mg. of bound phosphate/ml.; cozymase solution (corresponding to 5–20 γ of pure coenzyme); and water to a total volume of 3.0 ml. There is no advantage in tipping the cozymase from the sidearm. The CO<sub>2</sub> formation begins rapidly and assumes and maintains a constant rate for 1–2 hours. An example is given in Fig. 1. Unknown preparations are evaluated by comparison with standard cozymase samples used in the same experiment.

Stabilization of the apozymase is possible in various ways. Storage in the frozen state will preserve sufficient activity for about 24 hours. Preparations which will be stable for about two weeks can be prepared by spreading the fresh apozymase on glass plates in a layer 1–2 mm.



thick so that rapid drying with a fan will give a brittle material that is easily scraped off. The dry material obtained by rapid evaporation in the frozen state is also stable for about two weeks. In preparing the suspension from the dry product, 3.0 ml. of water plus the above listed ingredients should be added to 1 g. of dried apozymase.

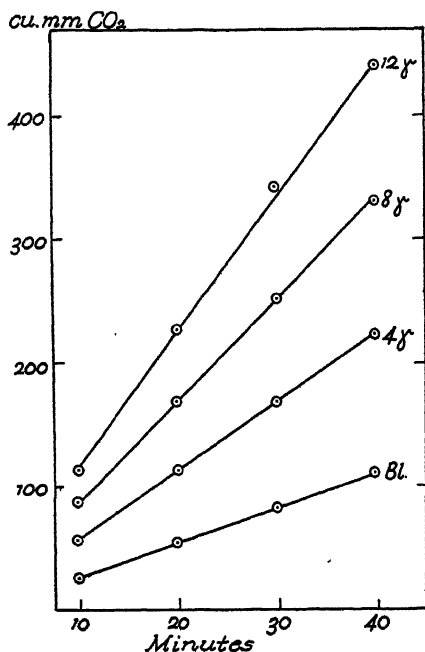


Fig. 1. Fermentation Test with Apozymase. (Experimental conditions are given in the text.)

It is not possible to prepare apozymase by this method from baker's yeast or English (top) brewer's yeast. W. M. Govier (17) has reported a modified method which may be resorted to if no dried bottom yeast is available. Another method for utilization of baker's yeast has been given by Neuberg (18).

The preparation of apozymase is a rather empirical process since there are considerable differences between yeast samples from different sources and sometimes even between batches obtained from the same source. The pH optimum for apozymase fermentation is between 6.0 and 6.4 and the phosphate concentration should be between 0.05 and 0.1 *M* (see Table I).  $Mg^{++}$  and  $Mn^{++}$  exert a very marked influence

TABLE I

*Influence of Phosphate Concentration on Fermentation Rate with Apozymase*  
(Experimental conditions as given in the text.)

Molarity of phosphate:	0.25	0.15	0.1	0.075	0.05	0.025	0.015
Cu.mm. CO <sub>2</sub> formed in 20 minutes:	197	268	306	320	309	232	186

on the fermentation rate.  $Mg^{++}$  is removed from some dried yeast preparations so readily by washing that, unless it is added to the apozymase, the fermentation remains very low. A molarity of the medium with respect to  $Mg^{++}$  of 0.005–0.01 is suitable. By adding  $Mn^{++}$  to this mixture in similar concentration a further increase of the fermentation rate can be achieved. Adenosine-5'-phosphoric acid is usually present in apozymase in sufficient amounts. The most extensive studies on apozymase fermentation have been carried out by R. Nilsson who has reported recently his results in a very comprehensive manner (13).

The fermentation test is valuable for the determination of cozymase in crude extracts, and is a rapid method for the comparison of preparations in any state of purity with a standard cozymase preparation.

*Other Biochemical Tests.* Jandorf, Klemperer and Hastings (19) have proposed the use of glycolytic reactions for the measurement of the activity of cozymase. An adaptation of the Thunberg methylene blue technique is used by Sumner and coworkers (8), and reference should be made to the enzyme spectrophotometry developed by Warburg (20).

*Determination of Growth-Promoting Properties in Microbiological Tests.* The discovery by Lwoff (21) that the nicotinamide nucleotides are the growth factor for *Hemophilus influenzae* and *H. parainfluenzae* (formerly termed "factor V") made possible the estimation of extremely small amounts of coenzyme. The outcome of the tests, however, depends on experimental conditions, and the method is rather delicate as has been emphasized by Gingrich (22). Other methods include the utilization of nicotinamide as a vitamin by a large number of microorganisms, as recently reviewed by Peterson (23). Comparative studies on the activity of the nicotinamide nucleotides as substitute for free nicotinamide are often missing. Considerable time and experience are necessary to establish the microbiological tests; hence, they are used mainly in laboratories where these techniques are a permanent feature.

*Testing for Contamination by Adenosine-5'-Phosphates.* These compounds can be discovered in cozymase preparations by their ability to activate the enzymatic liberation of phosphoric acid from phosphopyruvic acid. Experimental conditions for this test have been described by the authors (24).

### CHEMICAL TESTS

*Elementary Analysis.* Data corresponding to the formula:  $C_{21}H_{27}O_{14}N_7P_2$  are obtained only after drying *in vacuo* at 80–100°C. If the final precipitation of cozymase with alcohol and ether has been carried out in the presence of hydrochloric acid, the phosphoric acid group neutralizing the pyridinium nitrogen is replaced by chloride and the composition of the product is  $C_{21}H_{28}O_{14}N_7P_2Cl$ . Contamination with small amounts of adenylic acid is not revealed by elementary analysis due to the similarity in elementary composition.

*Titration.* Cozymase requires one equivalent of alkali for neutralization. Deviations from this may be due to the presence of hydrochloric acid (see above) and the adenylic acids particularly.

*Spectrophotometric Determination of Dihydrocozymase, Monohydrocozymase, and Cozymase.* The absorption maximum of cozymase at 260  $m\mu$  is not very characteristic, since all nucleotides, particularly the adenylic acids, show strong absorption in this region. For quantitative determination of the nicotinamide nucleotides the absorption maximum at 340  $m\mu$  of the dihydro derivatives is used. The experimental conditions for reduction with sodium hydrosulfite have been described by Warburg and coworkers (4); Drabkin and Meyerhof have recently reviewed this subject in detail (25).

For laboratories which are not equipped to use the ultraviolet absorption technique, measurements in the visible range can be carried out. E. Haas (26) recommends for this the reduction of 2,6-dichlorophenol-indophenol by dihydrocoenzyme. The decrease of the color intensity of the dye is proportional to the amount of dihydronicotinamide nucleotide.

We have found the colorimetric determination of monohydrocozymase to be very simple and accurate enough for many purposes. Adler and coworkers (27) first observed the formation of a yellow derivative of cozymase in the process of hydrosulfite reduction. This compound can be stabilized by carrying out the reduction in an alkaline medium.

The product formed is a semiquinoid radical (monohydro cozymase) (28). It shows a broad absorption band with a maximum at 360–370  $m\mu$  which extends sufficiently into the visible range to cause a yellow color. This color can be measured with an ordinary photoelectric colorimeter. The color is stable, and amounts from 0.5–3.0 mg. of cozymase can be rapidly compared with standard preparations. The intensity depends somewhat on the experimental conditions, the hydro-sulfite concentration being particularly important. The procedure employed by us is as follows:

One ml. of cozymase solution plus water and 1.0 ml. of a 1% sodium bicarbonate solution is pipetted into a micro cuvette. A layer of paraffin oil (*ca.* 0.5 ml.) is placed on top of this mixture, and 1.0 ml. of 1% sodium hydrosulfite solution in 0.1 *N* NaOH is added from a pipette whose tip extends into the bicarbonate and cozymase solution. The development of the color is completed in 2 minutes and the intensity remains unchanged for 2 hours. There is good proportionality over a range of 0.5 to 3.0 mg. of cozymase. A blank without cozymase is used giving an almost negligible correction. Impure preparations sometimes show a yellowish tinge in alkaline medium. For correction a mixture of cozymase and bicarbonate is prepared to which 0.1 *N* NaOH without hydrosulfite is added. For the measurements we used a Klett photoelectric colorimeter with a blue filter.

*Catalytic Hydrogenation.* Catalytic hydrogenation of cozymase converts the pyridine base into the hexahydro derivative (4). Under suitable experimental conditions the adenine nucleus does not add hydrogen. Instead of the platinum asbestos recommended by Warburg, we used a colloidal palladium solution stabilized with gum arabic (29). Recently a colloidal palladium solution in polyvinyl alcohol (30) has become available which is incomparably superior to both the earlier catalysts. This solution we found to be stable for years; much smaller amounts are used; and the results are more uniform. An example is given in Fig. 2.

After filling the flasks and manometers with hydrogen previously washed with  $\text{AgNO}_3$ ,  $\text{KMnO}_4$ , and  $\text{H}_2\text{SO}_4$ , the catalyst is almost saturated with the gas after one hour. When the gas uptake is less than 5 mm.<sup>3</sup>/15 minutes, the substrate is tipped from the bulb. Depending on the amount and the nature of the compound, the hydrogenation will be completed after 30–60 minutes. As can be seen from the experiment of Fig. 2, adenylic acid does not take up significant amounts of hydrogen. Thus, the presence of adenylic acid derivatives in cozymase preparations will not interfere. The accuracy of the method is limited only by the small errors of the manometric method. One  $\gamma$ -mole of

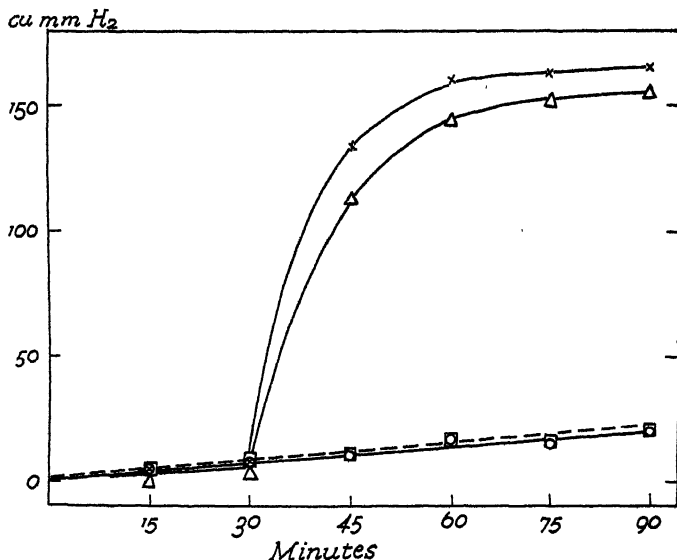


Fig. 2. Catalytic Hydrogenation of Cozymase. Each flask contained 1.0 ml. Pd catalyst,<sup>2</sup> 0.5 ml. 0.1 *M* borate buffer, pH 9.2; and the compound to be hydrogenated in the side arm, dissolved in 0.5 ml. borate buffer; *t* = 38°C. —○—○—Blank experiment. —□—□—Adenosine-5'-phosphate (2.55  $\gamma$ -moles). —△—△—Codehydrogenase I (2.05  $\gamma$ -moles). —×—×—Nicotinamide (2.30  $\gamma$ -moles).

nicotinamide-containing substance or of free nicotinamide should absorb 67.2 mm.<sup>3</sup> of hydrogen. The agreement between the values found and the theory is very satisfactory. Free nicotinamide which would simulate a high purity of preparations will hardly be encountered in cozymase preparations since the last step in all preparatory procedures consists of precipitation and washing with alcohol in which nicotinamide is soluble. Preparations which have been stored over a long period of time (see p. 132) are an exception to this. Other hydrogen absorbing compounds are lost early in the course of preparation (4). The hexahydrocozymase resulting from this treatment should not be mistaken for the biologically active dihydrocozymase.

*Colorimetric and Fluorometric Determination of Nicotinamide.* Extensive consideration has been given to the determination of nicotinamide and nicotinic acid in connection with vitamin studies. Most widely used is the reaction with BrCN which was discovered by König

<sup>2</sup> "Baker Colloid 46," Baker and Co., Newark, New Jersey.

(31). The base has to be split off from cozymase to give the reaction (32). The modifications of the test are too numerous to be listed here in detail; for recent literature see (33). It is important that the difference in color obtained from nicotinamide and nicotinic acid be considered. The preferred modifications of this test are the ones in which the preliminary hydrolysis is extended beyond the point necessary simply to split the pyridine base from the coenzyme. In this way all of the nicotinamide present is transformed into nicotinic acid. The method recently described by Levitas *et al.* (34) also seems to be promising. Their method uses the fluorescence of the acetone condensation product of the pyridone derivative of cozymase.

In conclusion, the following procedure is recommended to those embarking on a program of cozymase preparation so that they may obtain a well-defined product. First of all, a reference standard should be established. For this any cozymase preparation in the dry state will be suitable. The purest fractions of a preparation can be selected by comparing them with this reference standard in either the apozymase or the glycolytic test, and these fractions in turn can be examined further by the tests described above to establish their exact purity. Random samples obtained for occasional work from other laboratories (including the authors' laboratory) should at least be checked by the quantitative determination of the nicotinamide present in them. Soaking the preparations with alcohol or acetone prior to the test will remove free nicotinamide formed by the deterioration of the cozymase preparation after storage for long periods. For the determination of the bound nicotinamide, catalytic hydrogenation, spectrophotometric evaluation of the absorption of the dihydro derivative at  $340\text{ m}\mu$ , or a colorimetric test with  $\text{BrCN}$  can be used. Cozymase preparations are never better than is indicated by their nicotinamide content. Nicotinamide-containing impurities are the least likely to occur in them.

### SUMMARY

1. Reference is made to recent progress in the purification of cozymase, and some suggestions are given to improve the procedures further.
2. Some difficulties in evaluating the purity of cozymase are pointed out. The more important test methods are listed.
3. Detailed directions are given for the apozymase test, catalytic hydrogenation, and photometric determination of monohydrocozymase.

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# Optical Isomerism and Antimetabolite Effect of the Sulfoxides Derived from Methionine<sup>1,2</sup>

Ernest Borek and Heinrich Waelsch

*From the Departments of Biochemistry, New York State Psychiatric Institute and Hospital, and the College of Physicians and Surgeons, Columbia University, New York*

Received February 17, 1947

## INTRODUCTION

It has recently been shown that the sulfoxide derived from DL-methionine (MSO) is an effective antimetabolite against glutamic acid in the metabolism of *Lactobacillus arabinosus* (and *casei*). L-Glutamic acid overcomes specifically the growth inhibition produced by the sulfoxide. The antimetabolite probably blocks the metabolic availability of the  $\gamma$ -carboxyl group, since even twice the amount of sulfoxide which inhibits bacterial growth completely in the presence of glutamic acid has no effect in the presence of equivalent amounts of glutamine. On the other hand MSO inhibits strongly in the presence of ketoglutaric acid and the inhibition can not be reversed by increased amounts of the keto acid (1, 2, 3).

All the work reported in the previous communications was carried out with the sulfoxide derived from DL-methionine (MSO). As MSO contains 2 centers of asymmetry—the carbon atom and the sulfur atom—it can exist as 4 optical isomers. It appeared of interest to study the effectiveness of the various isomers as antimetabolites.

We are indebted to Dr. T. F. Lavine of the Lankenau Hospital, who has developed the method of resolution of MSO (4) and kindly made some of the isomers available to us.

## EXPERIMENTAL

The L- and L-Allo-MSO were prepared by Dr. Lavine from L-methionine by resolution *via* the picrates of the diastereoisomers resulting from the peroxide oxida-

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<sup>1</sup> Dedicated to Professor Carl Neuberg on his 70th birthday.

<sup>2</sup> Supported by a grant from the Williams Waterman Fund of the Research Corporation.

tion of the amino acid.<sup>3</sup> According to Dr. Lavine, our sample of L-MSO contained 97% of the L-isomer on the basis of the optical activity ( $[\alpha]_D^{25} = -67^\circ$  in water), and that of L-Allo-MSO 91% of the L-Allo-isomer ( $[\alpha]_D^{25} = 85^\circ$  in water). The mixture of D- and D-Allo-MSO was prepared in our laboratory from D-methionine ( $[\alpha]_D^{30} = -20^\circ$  in 0.2 N HCl). The D-methionine was made from S-benzyl-D-homocysteine ( $[\alpha]_D^{30} = -24.8^\circ$  (5)). Since the sulfoxide was obtained from D-methionine in almost theoretical yields, no appreciable resolution of the diastereoisomers could have taken place.

As before, the broth, synthetic medium and bacteriological techniques described by Hac, Snell and Williams (6) were employed. Since we had found that the extent of inhibition of bacterial growth is dependent on the size of the inoculum (3), it was carefully controlled.

The comparison of the antimetabolite activities of the MSO isomers was carried out at a glutamic acid concentration of 150 gamma/2.5 ml. ( $0.42 \times 10^{-3} M$ ), since a greater constancy of the antibacterial index (A.I.) was found at lower concentrations of glutamic acid. (A.I. at glutamic acid concentration of 0.2, 0.42 and  $0.82 \times 10^{-3} M$  was 30, 30 and 45, respectively.) All antimetabolite ratios reported in this paper were calculated from the minimal concentration of antimetabolite needed for complete inhibition of bacterial growth. The concentration of the mixture of sulfoxides derived from DL-methionine at complete inhibition was determined simultaneously with the isomer under test in 4 of the 6 representative experiments reported in Table I. The small daily variation of these concentrations (12, 12, 14,  $12 \times 10^{-3} M$ ) makes a quantitative comparison of the activities of the isomers possible.

## DISCUSSION

The data reported in Table I show that the diastereoisomeric sulfoxides derived from L-methionine are more potent than those derived from the D-antipode. This observation is similar to the finding that D-pantoyltaurine is more active than the analogue derived from the unnatural lactone (7).

In column 7 of the Table, the contributions of the isomers to the activity of MSO are calculated from their independent activities and their mole fraction in MSO assuming no asymmetric oxidation. The sulfoxide derived from D-methionine was not resolved into its two diastereoisomers since its low antimetabolite activity precludes an

<sup>3</sup> The designation of the different isomers of MSO was patterned after Rule 6 of the Committee on Nomenclature, Spelling and Pronunciation of the American Chemical Society (Chem. and Eng. News 25,1364 (1937)). The laevorotatory isomer of MSO derived from L-methionine has been arbitrarily named L-MSO and the dextrorotatory diastereoisomer L-Allo-MSO.

TABLE I  
*Activity of MSO Isomers as Antimetabolites*  
 (L-glutamic acid ( $0.42 \times 10^{-3}$ ))

MSO	Concentration at complete inhibition			A.I. <sup>1</sup>	Relative activity i-MSO = 100	Contribution in i-MSO
	$M \times 10^{-3}$	$M \times 10^{-3}$	$M \times 10^{-3}$			
i <sup>2</sup>	12	12	14 12	30	100	100
L	4.8 4.8			12	250	63
L-Allo-		12 12		30	100	25
D-			50 48	117	26	13 <sup>3</sup>
D-Allo- }						101

<sup>1</sup> A.I. = Antibacterial Index.

<sup>2</sup> i-MSO = the mixture of sulfoxides derived from DL-methionine.

<sup>3</sup> No attempt was made to correct this value for any asymmetric oxidation of the D-methionine to the D- and D-Allo-isomers, since it would not change the result beyond the experimental error (4) and a similar asymmetric synthesis may have taken place in the formation of the sulfoxide derived from DL-methionine.

accurate quantitative comparison of the isomers. It seems that the antimetabolite activity of MSO is the sum of the independent activities of the component isomers which evidently do not compete with each other for the susceptible enzyme system despite the large differences in activity.<sup>4</sup>

Since MSO contains two centers of asymmetry the study of the component isomers offers the possibility of evaluating the contribution of each of the asymmetric atoms to the antimetabolite effect. The two diastereoisomeric sulfoxides derived from L-methionine have a relative activity of 1:2.5 whereas the sulfoxides derived from D- and L-methionine (D- and D-Allo-; L- and L-Allo-) have a calculated ratio of approximately 1:7 (see Table). Therefore it appears that, for the antimetabolite activity of MSO, the configuration of the carbon is of greater importance than that of the sulfur. This is of interest since MSO is an antimetabolite against glutamic acid, but not against glutamine, and therefore MSO interferes with the metabolic availability of the  $\gamma$ -carboxyl group.

<sup>4</sup> In another experiment the activity of the sulfoxide derived from a commercial sample of L-methionine (containing 80% L-form) was tested. The activity corrected for the presence of D-form but not for asymmetric oxidation amounted to 79 (MSO = 100), a value short by 10% of the sum of the separately measured activities of the L- and L-Allo-isomers.

The importance of the  $\alpha$ -carbon for the antimetabolite activity of MSO is shown by the irreversibility of the inhibition in the presence of large amounts of ketoglutaric acid which can substitute for glutamic acid in the nutrition of the bacteria.

The difference in activity of the diastereoisomers derived from L-methionine (L- and L-Allo-) indicates that the enzymes involved discriminate between the two isomers originating from the asymmetry of the sulfur atom, even though the methylsulfinyl grouping apparently interferes with the  $\gamma$ -carboxyl group of glutamic acid which has no center of asymmetry.

The evidence presented indicates that the asymmetry of elements other than carbon may have biological significance (see 8). Since we have shown that the antimetabolite effect of MSO is easily overcome by the organism, probably by reduction to methionine, it may be argued that the different potency of the isomers is a measure of their susceptibility to reduction. Even if this were the case, the biological discrimination between the two forms of asymmetric sulfur by the reducing system would be of considerable interest. But the greater ease of reduction of the sulfoxides from D-methionine is not likely since available evidence points to the preferential utilization of L-methionine by microorganisms (9, 10).

#### ACKNOWLEDGMENTS

We are indebted to Mrs. Phyllis Owades and Mr. Robert Gittler for their able assistance during the course of this work.

#### SUMMARY

The antimetabolite activity of the component isomers of the inactive sulfoxide derived from methionine was studied. The sulfoxides derived from L-methionine are more active than those derived from the unnatural antipode. The diastereoisomers derived from L-methionine have significantly different activities indicating enzymic sensitivity to the asymmetry of the sulfur atom.

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# Physical and Chemical Properties of Crystalline $\alpha$ -Amylase of Hog Pancreas<sup>1</sup>

Kurt H. Meyer, Ed. H. Fischer and P. Bernfeld

*From the Laboratoires de Chimie Organique et Inorganique de l'Université,  
Geneva, Switzerland*

Received April 1, 1947

We recently described the isolation (1, 2) and crystallization (3) of  $\alpha$ -amylase from hog pancreas. The crystalline enzyme was obtained in eight steps from the crude extract of the dry defatted gland in a yield of 12%. Amylase represents about 4.5% of the protein content of the crude extract. The enzyme crystallizes readily from a 4–5% aqueous solution, in prisms or in fine needles (Figs. 1, 2 and 3).

After two recrystallizations the mother liquor exhibits the same degree of purity (ratio of activity to N content) as the crystals themselves (Table I). Electrophoresis at various pH of the crystallized product shows a single component (Fig. 4). The crystals must, therefore, be considered as pure  $\alpha$ -amylase.

The enzyme gives the typical protein reactions, its composition is:<sup>2</sup>

45.6% C   6.60% H   16.0% N   0.0% S   0.6% P   1.3% Ash

TABLE I

	Yield of crystalline product	Degree of purity*	
		Crystals	Mother liquors
	<i>per cent</i>		
1st cryst.	80	$3.8 \times 10^3$	$2.1 \times 10^3$
1st recryst.	75	$4.0 \times 10^3$	$3.6 \times 10^3$
2nd recryst.	80	$4.0 \times 10^3$	$4.0 \times 10^3$

\* Mg. maltose/mg. Kjeldahl N.

<sup>1</sup> Dedicated to Professor C. Neuberg on his 70th birthday.

<sup>2</sup> Microanalyses were carried out by the analytical laboratory of the University of Geneva (dir. Prof. P. Wenger).





FIG. 1. 220 X.

FIG. 2. 220 X.  
Crystalline  $\alpha$ -amylase

FIG. 3. 100 X.

The aqueous solution of the pure enzyme is very stable and does not lose any activity after one month at 2°C. at pH 6.9. However, an enzyme still containing 60% of impurities may lose as much as 70% of its activity in 24 hours under the same conditions.

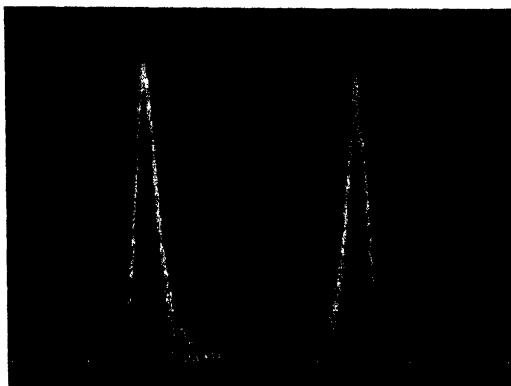


FIG. 4. Electrophoretic diagram of crystalline  $\alpha$ -amylase. Optical system: Philpot-Svensson. Buffer: Michaelis pH 6.5;  $\mu = 0.1$ . Temp.: 4.0°C. Time: 7,320 sec. Electrical field: 2.74 volts-cm.<sup>-1</sup> Descending boundary at the left.

The solubility of the pure product in neutral water is 0.22% at 2°C. and 1.0% at 25°C. The molecular heat of solution deduced from these values is about 10,000 cal./mole. Addition of NaCl, Na<sub>2</sub>SO<sub>4</sub>, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, MgCl<sub>2</sub> and sodium glycerol phosphate up to concentrations of  $M/30$  has no marked effect on the solubility in a phosphate  $M/100$  buffer solution of pH 6.9. At pH 8.4 (0.1  $N$  NH<sub>4</sub>OH) the solubility in water is increased to 3–4% at 2°C.

The electrophoretic mobilities  $u$  are:

$$\text{at pH 7.9 } (\mu = 0.1) \ u = 3.1 \times 10^{-5} \text{ cm}^2 \cdot \text{sec}^{-1} \text{ volt}^{-1}$$

$$\text{at pH 6.5 } (\mu = 0.1) \ u = 1.8 \times 10^{-5} \text{ cm}^2 \cdot \text{sec}^{-1} \text{ volt}^{-1}$$

On application of Longworth's formula, the diffusion constant  $D$  can be obtained from electrophoretic diagrams. At various pH's,  $D$  is between 12 and  $12.5 \times 10^{-7} \text{ cm}^2 \cdot \text{sec}^{-1}$ , calculated for 20°C. and buffer solution. Such high values for  $D$  have been found for proteins of very low molecular weight, in general less than 20,000.

The light absorption of the aqueous solution shows a maximum at  $280\text{ m}\mu$  and a small peak at  $291\text{ m}\mu$  (Fig. 5).

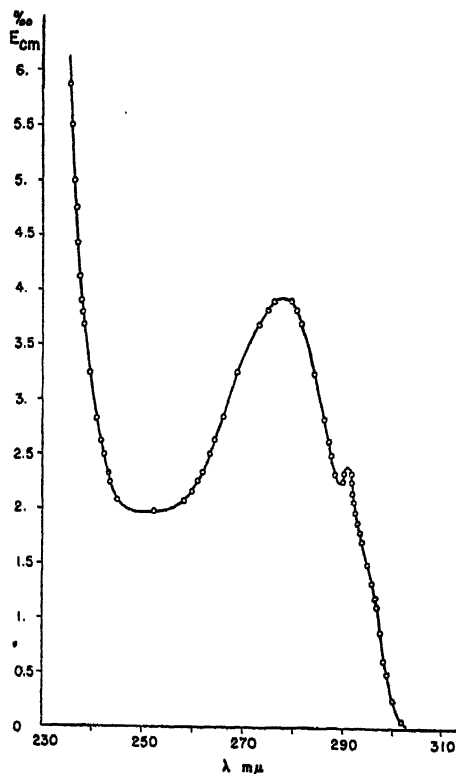


FIG. 5. Light absorption of aqueous solution of  $\alpha$ -amylase.

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# The Hydrolysis of Alginic Acid with Formic Acid <sup>1</sup>

H. A. Spoehr

*From the Division of Plant Biology, Carnegie Institution of  
Washington, Stanford University, California*

Received March 24, 1947

Among plant products, alginic acid is one of the most difficult to hydrolyze. This is due to the fact that it is split into its components only very slowly and also because its product of hydrolysis easily undergoes decomposition under the conditions of acid concentration and temperature which commonly have been used to effect hydrolysis. In spite of these difficulties Nelson and Cretcher (1) and Bird and Haas (2) were able to demonstrate that alginic acid yielded D-mannuronic lactone on acid hydrolysis. Schoeffel and Link (3) worked out methods for obtaining the free D-mannuronic acids and the lactone from the products of hydrolysis, albeit in low yields, and established the properties of these compounds. Recently, Frush and Isbell (4) have described a method in which better yields of D-mannuronic acid lactone can be obtained from the hydrolysis of alginic acid. In all of these investigations the hydrolysis of the alginic acid was accomplished by the use of strong mineral acids. By the methods thus far employed it is necessary to neutralize the mineral acid used for hydrolysis. The process is rather cumbersome, and where products free from traces of inorganic salts are desired, as for certain biological tests, it involves repeated purification. The method of hydrolysis here described obviates the necessity of removing the acid used for hydrolysis through neutralization and the possible losses entailed through adsorption on the inorganic salts. It also avoids the danger of enolization of uronic acids to keto acids. The hydrolysis of alginic acid is given as an example of the application of formic acid for purposes of hydrolysis of compounds of this nature. Inasmuch as preliminary tests with other polyuronides, polysaccharides and a number of polymeric compounds of plant origin have given evidence of their being hydrolyzed by this method it may prove to have wider usefulness.

<sup>1</sup> Dedicated to Professor Carl Neuberg on his 70th birthday.

## PREPARATION OF D-MANNURONIC ACID LACTONE

It was found advantageous to have the alginic acid thoroughly dry and as finely divided as possible. For this purpose it was dried *in vacuo* over calcium chloride for a week and then ground in a pebble mill with agate marbles for about 200 hours. Some preparations of alginic acid have a fluffy, cotton-like texture. These cannot be ground conveniently and are put through a Wiley mill. The finely divided material was stored in a well-sealed container.

Fifty g. of the ground alginic acid is added, in small amounts, to 800 ml. of 90% formic acid in a round bottom flask with glass joint. After each addition of the alginic acid the mixture is thoroughly shaken to obtain as complete a suspension as possible and to avoid formation of lumps. The flask is connected with a reflux condenser, with glass joint, the upper end provided with a calcium chloride tube, and the mixture boiled for 10 hours. In the early stages there is a tendency of the mixture to bump which can be relieved by occasional shaking and the introduction of a few glass beads. During the course of the boiling the mixture becomes very dark. It was found that the darkening is less when the hydrolysis is carried out in an atmosphere of carbon dioxide, but there appears to be no advantage in quantity or quality of yield by following this procedure. The suspended alginic acid dissolves during the course of the first few hours of boiling. It should be mentioned that in separate experiments no evidence was obtained of the liberation of carbon dioxide from the boiling solution of alginic acid in formic acid. This fact should indicate that the uronic acid is not decarboxylated under these conditions in contrast to boiling in mineral acid solutions.

The formic acid is removed by distillation at reduced pressure at 50–55°C. in an apparatus with glass joints. The distilled acid can be used again for hydrolysis with equally good results. The residual gum is dissolved in 100 ml. of 95% ethanol which is distilled off at reduced pressure for the purpose of removing the formic acid held by the gum. Occasionally it is advisable to repeat this step. The gum is dissolved in 1,000 ml. of 95% ethanol and treated with decolorizing carbon. The filtrate from the carbon is concentrated at reduced pressure, 50°C., until no more solvent distills. The residual gum is dissolved in 12 ml. of absolute ethanol, with gentle heating, and set aside for crystallization. This usually occurs in about 12 hours after scratching with a glass rod. The crystals are finally allowed to grow for 2–3 days in the

refrigerator, when the entire mass solidifies. The crystals are triturated with a small amount of a mixture of absolute ethanol and acetone, 30:70 by volume (3). They can then be filtered and are washed with a small amount of the same ethanol-acetone mixture and dried *in vacuo* over calcium chloride.

The first crop of crystals amounts to about 30% of the alginic acid used for hydrolysis. A small second crop can be obtained from the filtrate by distilling off the solvent and dissolving the residual gum in a small quantity of ethanol. It is, however, more profitable to treat the residual gum again with 15 times its weight of formic acid and to boil this solution for 8–10 hours. The same procedure is followed as in the first hydrolysis, and a second crop of crystals of D-mannuronic acid lactone is obtained, amounting to an additional 9% of the original alginic acid used. A third hydrolysis, following the same procedure, yields another 6%, making a total of about 44% of the alginic acid which is obtained as D-mannuronic acid lactone. From 8 preparations the melting point of this product ranged from 138–140°C. and the  $[\alpha]_D^{18}$  from +86.90° to +87.09°.

The D-mannuronic acid lactone can be purified by dissolving it in water, treating with a small quantity of decolorizing carbon, and concentrating at reduced pressure to a thick gum, which crystallizes almost immediately. These crystals are triturated with a little ethanol-acetone (30:70) and are filtered and dried. These crystals have a m.p. of 145°C. (corr.) and  $[\alpha]_D^{18} = +92.13^\circ$  (after 40 minutes with 3.104 g. in 100 cc. water).

#### SUMMARY

Alginic acid can be hydrolyzed with 90% formic to yield about 45% of D-mannuronic acid lactone. The use of formic acid instead of mineral acids offers some advantages for the hydrolysis of various natural products.

A word of warning on the care required in handling concentrated formic acid may not be amiss.

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# Studies on the Fermentation of Cigar Leaf Tobacco<sup>1</sup>

## I. Nature of the Fermentation; Losses of Solids; Increase of Insoluble Solids

Walter G. Frankenburg

*From the Research Laboratory of the General Cigar Co., Inc.,  
Lancaster, Pa.*

Received April 17, 1947

### INTRODUCTION

The chemical changes in harvested tobacco caused by the processing of the leaves have been studied repeatedly. A thorough knowledge of these changes promises to be not only of interest to the tobacco industry, but also to give general information on the chemical nature and on the reactivity of the components of the tobacco leaf in its post mortem state. Whereas the chemistry of the initial phase, the drying of the freshly harvested tobacco leaves ("curing") has been studied in many laboratories (1), relatively little work has been done on the chemical aspects of the later stages in the industrial treatment of tobacco leaves, usually called *Fermentation*<sup>1a</sup> or *Sweat*. Furthermore, most of the studies in tobacco chemistry have been directed to the analytical determination of a few characteristic components of the leaf tissues such as the alkaloids, amino compounds, carbohydrates, and organic acids such as citric and malic acids. To the remainder of the less known, and analytically less accessible, components but little attention has been given, except the valuable information on this neglected sector gathered by C. Neuberg (2) and his coworkers, and by some others (3).

In order to gain a more complete picture of the chemical composition of tobacco leaves and of the transformations which occur in the leaves,

<sup>1</sup> Dedicated to Professor Carl Neuberg on his 70th birthday.

<sup>1a</sup> The term "Fermentation" should not imply that any process similar to the process of alcoholic fermentation occurs in tobacco, nor does it mean that microorganisms are necessarily involved in it.



additional work is required. Once the main chemical components contained in the leaves are known qualitatively and quantitatively, it will be easier to discover the kind and extent of reactions taking place between them during the various stages of the tobacco processing. Specifically, the identification of analytically accessible reactions in the leaf tissues as characteristic features of the fermentation process will facilitate a systematic approach to the repeatedly discussed question of whether the driving factors in the fermentation of tobacco are chemical (catalytic) in nature, enzymatic in character, or finally, due to bacterial influences. So far, most of the work done to decide the latter question was based on the establishment of conditions either favorable or unfavorable to enzyme action or to bacterial action, followed by the purely qualitative evaluation of whether or not a fermentation of the leaves could be discovered by visual inspection.

### THE PRINCIPLE OF FERMENTATION OF LEAF TOBACCO<sup>2</sup>

The purpose of the fermentation of tobacco is to improve its smoking properties beyond the point reached after its curing. Often a storage period of many months ("natural sweat") is interposed between the curing and the fermentation, and it is likely that during this time of exposure to the fluctuations of temperature and moisture, considerable chemical change occurs in the leaf tissues.

The kind of fermentation process applied to the leaves after these preliminary stages depends on the type of the processed tobacco. In its mildest form ("aging") the fermentation consists of a prolonged storage of the slightly moistened (10–12% moisture), packed leaves at normal temperatures; it is commonly applied to certain (flue cured) types of cigarette tobacco. In its most drastic form ("sweat") the fermentation involves the moistening of the cured tobacco leaves with definite, often considerable amounts of water (about 10–40% of the tobacco weight),<sup>3</sup> the packing under pressure of the moistened leaves in containers, and the storage of these containers in rooms at about 45°C. and  $\pm 60\%$  relative humidity.

This drastic "sweat" or "Resweat" process is applied to certain cigar filler types such as Pennsylvania Seedleaf tobacco (U. S. Type #41). The storage of the tobacco in the heated rooms is periodically interrupted by unpacking and airing the leaves. These interruptions are normally applied when the spontaneous temperature in-

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<sup>2</sup> A more detailed description of the various types of fermentation is presented by W. W. Garner. See (4).

<sup>3</sup> The amounts of moisture added to the leaves by their controlled immersion in water ("casing") is, in a well conducted fermentation process, determined individually for every batch of tobacco, according to its color, texture, softness and smell.

crease of the fermenting tobacco has reached its peak. An easily fermenting tobacco requires only one or two of these interruptions whereas a very tough or raw tobacco has to be subjected to up to ten or twelve airing operations during its sweat. A careful observation of the spontaneous temperature increases combined with repeated thorough visual inspections of the tobacco during the fermentation, permits the control of its progress toward the desired final state.

At the end of a proper fermentation of cigar filler tobacco, the leaves have lost the initial "raw" odor and have assumed an aromatic, partly ammoniacal, smell; the color has changed to a slightly duller shade, the surfaces have lost the initial glossy appearance and their sticky and gummy feel, and the texture has become considerably more tender and brittle. On ignition, well fermented leaves develop an aromatic smoke which is free of the pungency of the smoke of unfermented tobacco leaves.

#### ANALYTICAL STUDIES OF THE FERMENTATION

All the following results relate to the fermentation of *Pennsylvania Seedleaf Tobacco*. Analytical determinations<sup>4</sup> were made for different crops of tobacco, every single crop being represented by at least thirty samples of various origins. Each of these samples was subjected to analyses before and after fermentation. This permits the determination of the very pronounced individual variations from sample to sample of any given crop. Furthermore, an averaging of the results for every single crop gives information on the characteristic variations from crop to crop. The compilation of many data of this kind may finally help to establish characteristic and permanent chemical features of the fermentation of Pennsylvania tobacco.

#### *Oxidative Reactions*

The spontaneous heating of fermenting tobacco leaves indicates the occurrence of exothermic reactions in the leaf tissues. Experiments with samples of a few leaves in air-tight thermostatically controlled containers proved that properly moistened tobacco undergoes no fermentation, even at elevated temperatures, in a nitrogen or hydrogen atmosphere. In air or oxygen, however, fermentation sets in. The consumption of considerable amounts of oxygen is clearly demonstrated

<sup>4</sup> A short description of the experimental methods is given on p. 177 *et seq.*

by the creation of a partial vacuum in the containers. Obviously, there is no immediate evolution of equivalent amounts of carbon dioxide after the oxygen has been absorbed by the leaves.

Quantitative studies of the gas exchanges and of the formation of water as a reaction product, as well as calorimetric measurements of the total heat of reaction would be very desirable. The proper execution of experiments of this kind is complicated because it is difficult to begin and to maintain a regular fermentation of small leaf samples under the artificial conditions existing in air-tight containers, and also because the heat of reaction is evolved over a long period of time during which simultaneous heat losses by convection and radiation occur.

There is, accordingly, no doubt that exothermic oxidations of some of the leaf components play an important part in the fermentation process.

### *Losses of Solids*

If carried far enough every oxidation of organic compounds leads to water and carbon dioxide as the final end products with ammonia often being produced as the fragment of oxidized (or hydrolyzed) nitrogenous substances. Appreciable amounts of carbon dioxide and ammonia are actually evolved during the fermentation of tobacco. How much of the water given off originates from the oxidation of organic compounds is difficult to determine due to the presence and slow evaporation of the initial moisture content of the leaves. The partial or total oxidation of some of the organic leaf constituents is further indicated by a *loss of total solids* at the end of the fermentation (AF) compared with the weight prior to fermentation (BF). There seems to be no doubt that the preponderant part of these weight losses originates from the evolution of gaseous products such as carbon dioxide and ammonia and of vapors such as water and volatile organic compounds (*e.g.*, small amounts of amines and alkaloids). An additional, not negligible, source of the decrease of weight is due to the direct loss of small amounts of solids which come off in the form of dust or small particles as the breakdown products of gummy substances which coat the leaf surfaces. The quantities of solids lost in this way appears to be small compared with the weight losses caused by gasification and evaporation, but we shall see that the analytical determinations of some inorganic components leave no doubt as to the existence of this second type of loss.

*Determination of Weight Losses.* There are two different methods of determining the loss of solids during the fermentation. The *direct method* involves the weighing of large units of tobacco before and after the fermentation, with simultaneous determinations of the moisture contents at both stages, in order to evaluate the "dry weights" at both stages and with a simultaneous registration of, and correction for, the purely mechanical losses of tobacco fragments incurred during its handling. The *indirect method* is based on the assumption that certain substances remain, under all conditions, unchanged during the fermentation. Obviously, the percentage of these substances is bound to show an apparent increase in the fermenting tobacco due to the decrease of its total weight. Inorganic substances are most likely to remain constant. Calcium and potassium are the inorganic elements most abundant in tobacco leaves; their analytical determination is fairly dependable. Accordingly, they lend themselves as indicators for the indirect method. In the course of our studies, we recognized that other inorganic constituents such as iron, phosphorus, manganese and silica are not only less suitable as indicators because of their smaller amounts in the leaves, but also because they seem to be implicated in the losses of material from the leaf surfaces. Of the organic compounds, only very resistant substances such as the "crude fiber" (mainly cellulose) may come into consideration as a potential basis for the indirect determination of weight losses.

*Magnitude of Weight Losses.* The following data on weight losses derived from our experiments are based on many hundreds of individual determinations. For every tobacco sample the determinations were made in duplicate, and repeated whenever the deviations in a given pair of values exceeded the limits of the analytical and of the sampling errors.<sup>5</sup> Instead of presenting the voluminous tables showing the results obtained with individual samples, we shall record here only the average values for a few crops.

Being the result of statistical evaluation, these averages show a fairly dependable picture of the weight losses of a given crop. They do not indicate, however, the relationships which possibly exist between the initial chemical composition of every individual tobacco sample and its response to the fermentation. As already mentioned, the individual samples of every crop represent a wide range of initial compositions and of individual capacities to undergo fermentation. It is of practical importance and scientific interest to find the relationships, if any, between these two properties. In this paper, such individual evaluations of the experimental data will not be discussed *in extenso*.

On the average, the solids lost during the fermentation of Pennsylvania Tobacco range from about 7% to 10% of the weight prior to the fermentation, as shown in Table I.

<sup>5</sup> See Ref. (1), p. 318.

TABLE I

*Average Values of Solids Lost During Fermentation, Determined  
by the Direct Method*

29 Samples of Pennsylvania Seedleaf Tobacco per crop.

Crop	Average loss of solids in % of dry weight before fermentation	
	For entire leaf	For leaf blades (calc'd.)*
1936	9.50	10.64
1939	7.65	8.40
1941	8.03	8.96

\* See p. 161.

In Fig. 1 is depicted a frequency distribution curve for two crops which demonstrates the wide range of weight losses of the individual samples. As a rule, the smallest weight losses were observed for the samples which show the least fermentation and *vice versa*.

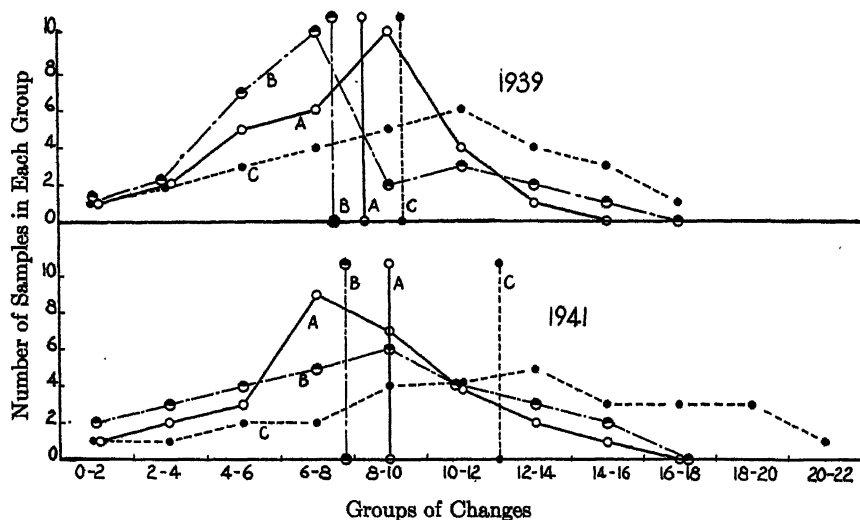


FIG. 1. Frequency distribution of the weight losses and of the apparent calcium and potassium increases caused by fermentation. 1939-Crop and 1941-Crop of Pennsylvania Seedleaf Cigar Tobacco, 29 samples each.

The limits of each group represent for Curve A: Decrease of weights of entire leaves, in *per cent* of their "Before Fermentation" (BF) weights; for Curve B: Apparent increases of calcium in leaf blades, in *per cent* of their BF calcium contents; for Curve

C: Apparent increases of potassium in leaf blades, in *per cent* of their BF potassium contents.

The vertical lines represent the averages of the three types of changes. For the sake of an adequate comparison of these averages, the average weight loss of the entire leaves has been converted to the average weight loss of the leaf blades (see Table I, last column).

The values obtained by the indirect method with calcium and potassium as indicators, columns 2 and 3 (Table II), confirm the order of magnitude of the values listed in Table I. They show, on an average, the apparent increase of the percentages<sup>6</sup> of calcium and potassium in the tobacco samples due to fermentation.

It is well to consider that all the individual values of the indirect method are calculated, *e.g.*, for calcium, as follows:

$$\frac{(\% \text{ Ca in fermented sample}) - (\% \text{ Ca in unfermented sample})}{(\% \text{ Ca in unfermented sample})} \times 100,$$

a formula in which every analytical and sampling error is greatly magnified. Hence, these indirect values possess, necessarily, only a limited accuracy. Nevertheless, there can be no doubt that calcium and potassium, possibly also manganese, and crude fiber are, in first

TABLE II

*Relative (Apparent) Changes of Certain Constituents in Leaf Blades of Pennsylvania Seedleaf Tobacco, Caused by Fermentation*

Crop (1)	Ca (2)	K (3)	Crude fiber (4)	Mn (5)	Fe (6)	P (7)	SiO <sub>2</sub> (8)
1936	—	—	+6.5	—	—	—	—
1939	+7.5	+9.3	—	+4.5	-1.1	+0.7	-1.2
1941	+7.9	+12.0	—	+10.0	-8.2	±0	-5.9

The values in this Table II are averages of 29 samples analyzed per crop. For every crop, these samples are identical with those referred to in Table I.

approximation, suitable indicators of the losses of sample weights. The values found for iron, phosphorus, and silica (Table II, columns 6 to 8), however, are obviously not applicable for this purpose. Instead of apparent increases of about 7-10%, the percentage figures of

<sup>6</sup> These percentages are all based on the dry weight of the samples at the stage at which they were analyzed.

these substances show, on the average, only very small increases, or even decreases, as a result of the fermentation. The simplest explanation for this discrepancy is that considerable parts of the materials containing these elements were somehow lost during the fermentation.

As it is extremely unlikely that these decreases of some inorganic elements are due to volatilizations, one has to conclude that roughly 10% or more of the iron-, phosphorus- and silica-containing substances come off the fermenting leaves together with the residues of gum and wax coatings mentioned previously. Considering that the soil as well as the fertilizers used for growing tobacco contains  $\text{SiO}_2$  (as sand)  $\text{Fe}_2\text{O}_3$  (soil ingredient) and  $\text{P}_2\text{O}_5$  (part of phosphate fertilizers), it is not improbable that foreign particles containing these elements were embedded in, and sticking to the gummy leaf surfaces. With the removal of the surface gums and waxes in the course of the fermentation some of these foreign particles will also disappear.

A few further observations support this assumption. Practically all the silica found in the leaves consists of sand granules. Only a small fraction (8-10%) of the total iron of the leaves is in a water-soluble form.<sup>7</sup> Addition of dissolved iron salts containing only 10-20% of the analytically-found total iron produces a dark brown to black coloration of the leaves, a further qualitative proof that most of the analytically-found total iron is present in a form which is not soluble in the sap of the leaves.

*Complications in Determining the Weight Losses.* The picture of the weight losses caused by the fermentation proves, on closer inspection, to be somewhat more complicated. The direct weight losses (Table I) were derived from the weight differences found for the *entire* tobacco leaves before and after the sweat. The analytical data serving as the basis for the indirect methods were, however, obtained from the *blades* of the unfermented and fermented tobacco leaves. The blades are the commercially used parts of the leaves, and are obtained by the removal of the midribs ("stems") from the fermented leaves. Considering that the chemical composition of the leaf blades differs considerably from that of the stems, it can be expected that both leaf parts respond, also, differently to the fermentation, and show different weight losses. Hence, the values found by the direct and by the indirect methods do not necessarily have to lead to identical figures.

<sup>7</sup> Found by analysis of water extracts of the leaves. The extracts were dried, ashed, and analyzed.

To gain information on this point, the blades and midribs of ten leaf samples (1941-crop) were analyzed separately, before and after fermentation of the entire leaves. The weight losses of the entire leaves of these ten samples averaged 9.5%. The calcium-contents in *per cent* of the blades and of the midribs were about equal before the fermentation. After fermentation, the blade-calcium showed an apparent increase of 12%, the midrib-calcium, only an apparent increase of 6%. If these apparent changes of the calcium-percentages are accepted as dependable indicators of the weight losses of both leaf parts,<sup>8</sup> and if it is considered that the average ratio of midrib-weight to blade-weight is about 1:2.5,<sup>9</sup> it must be concluded that the overall weight loss of the *entire* leaf amounts to  $\frac{(2.5 \times 12) + 6}{3.5}$  or to 10.3%, in fairly good agreement with the average weight loss of 9.5% found by the direct method for the entire leaves of the same samples.

Still more complicated are the conditions prevailing for *potassium*. In the analyses of midribs and blades, the potassium contents of the blades and midribs were found, before fermentation, to be 3.7% and 7.1%, respectively. After fermentation, the leaf potassium had increased to 4.3%, the midrib potassium had decreased to 6.5%. These changes are equivalent to an apparent potassium increase of as much as 16% in the leaf blades and a potassium decrease in the midribs of about 9%. The calculated overall change of potassium in the entire leaf is  $\frac{(2.5 \times 16) - 9}{3.5} = 8.9\%$ . This

detailed analysis for potassium seems to indicate that a part of the easily soluble potassium salts migrated, during the fermentation in the moist leaf tissue, from the midrib, as an area of higher concentration, into the blades.<sup>10</sup>

A migration of K salts into the leaf blades during fermentation may be the reason for the considerably higher K increases found for leaf blades by the indirect method compared with the weight losses of the leaf blades. (See the averages shown in Fig. 1.)

*Conversion of Percentages to a Common Basis.* Dependable quantitative information on the weight losses is important for the entire study of the chemical changes during fermentation. A satisfactory picture of the transformation of leaf components requires obviously a fairly

<sup>8</sup> This involves the assumption that any amounts of calcium lost together with the surface coatings are negligible. Fortunately, the amounts of calcium contained in the leaves are much larger than the order of magnitude of the surface losses of iron and phosphorus. This makes it probable that any "surface losses" of calcium can be neglected, in first approximation. Small surface losses of calcium are indicated by the fact that, as a rule, the Ca increases of the leaf blades are slightly lower than the weight losses of the leaf blades (Fig. 1).

<sup>9</sup> This ratio was derived from weighings of leaf blades and midribs. See also Ref. (5).

<sup>10</sup> Another fraction of the potassium salts might be leached out from the midrib by the water used in the casing operation. This may explain the calculated value of 8.9% against the directly determined loss of leaf weight of 9.5%.



accurate knowledge of the *absolute* changes of all these components rather than a comparison of their percentages based on the dry weights which decrease in the course of the processing. The actual transformations will only emerge clearly, if all the analytical values found are converted to one and the same common basis, preferably by expressing them as percentages of the initial "before-sweat" (BF) dry weight of the individual analyzed samples. To convert the percentage figures found after the fermentation (AF percentages) to BF percentages, the AF values must be diminished by the same fraction by which the total dry weight of the analyzed sample has decreased from its initial value to the stage at which the fermented tobacco is analyzed.

With a few exceptions, all our analyses, before and after fermentation, were made on the leaf blades rather than of the entire leaves. Accordingly, the correct conversion of the percentage of any leaf constituent to the common basis of initial dry weight requires, for every tobacco sample, the knowledge of the weight loss of its *leaf blades*. This loss is not identical with the value found by the direct method because the latter represents the weight loss of the *entire leaves*. The indirect methods are, to be sure, based on analyses of the leaf blades, and should, therefore, theoretically yield the weight losses of the blades and herewith the correct conversion factors. The limited accuracy, however, of the values found by the indirect method, together with the complications by migration effects, *etc.*, make it doubtful whether they can be safely recommended as the basis for sufficiently accurate conversions. Fortunately, the separate analyses of blades and midribs mentioned above show that, on an average, the weight loss of the blades exceeds the weight loss of the entire leaves by about 12%, without drastic deviations from this figure from sample to sample.<sup>11</sup> The blades lose, for instance, 11.2% of solids if the weight loss of the entire leaf amounts to 10%. Accordingly, all the conversions of percentage figures in our analyses of fermented tobacco leaf blades were converted to percentages of the initial weight of the blades by decreasing them by a value equal to 1.12 times the loss of the entire leaf weight (found by the direct method) of the sample investigated.

<sup>11</sup> This relationship is equivalent to the statement that (percentage weight loss of blades) : (percentage weight loss of entire leaves) : (percentage weight loss of midribs) = 1.12 : 1.10 : 0.64.

# CHANGES BETWEEN WATER-SOLUBLE AND WATER-INSOLUBLE FACTORS

## General Outline

Another effect which accompanies, as a characteristic feature, the fermentation of tobacco leaves, is a shift in favor of water-insoluble solids. The ratio of water-insoluble solids to water-soluble solids, found for a given tobacco sample by means of a standardized extraction method,<sup>12</sup> is considerably smaller before the fermentation (BF) of this sample than after the fermentation (AF). The magnitude of this change is illustrated by Table III, Nos. 1 and 2.

TABLE III

*Amounts of Water-Soluble and Water-Insoluble Solids, Before (BF)  
and After Fermentation (AF)*

Pennsylvania seedleaf tobacco; leaf blades, average of 30 representative samples per crop.

No.	Stage of processing	Amounts found expressed as	1939—Crop		1941—Crop	
			Soluble solids	Insoluble solids	Soluble solids	Insoluble solids
1	BF	<i>Per cent of BF dry weights</i>	41.8	58.2	45.8	54.2
2	AF	<i>Per cent of AF dry weights</i>	35.5	64.5	37.9	62.1
3	AF	<i>Per cent of BF dry weights</i>	32.5	59.1	34.5	56.5
4	AF	<i>Per cent of BF dry weights corrected for amounts lost</i>	38.9	61.1	41.5	58.5
5	Values No. 1 minus Values No. 4 Actual conversion of soluble to insoluble solids in <i>per cent</i> of BF dry weights.		2.9		4.3	

<sup>12</sup> See p. 178.

To see whether this effect corresponds to an actual conversion of soluble to insoluble solids, the AF values must be converted to the BF basis (Table III, No. 3), and the converted values, furthermore, must be corrected for the total losses of solids by the water-soluble fraction as well as by the water-insoluble fraction. The conversion to the BF dry weights (Table III, No. 3) has been discussed above. The correction for the amounts lost must be based on an estimate of how much of the total loss of dry weights resulting from the fermentation must be allocated to the solids contained in the water-insoluble fraction.

Even before any discussion of quantitative data, it can be expected that the main part of the weight losses during the fermentation originates from volatilizations and oxidative gasifications of the leaf components contained in the water-soluble fraction. This fraction comprises the lower molecular, more reactive compounds which are in an easily accessible form, and it is, therefore, reasonable to assume that those reactions which finally lead to weight losses will, in the first line, concentrate on components in the water-soluble fraction.

There are, however, indications that it would be incorrect to allocate the total weight losses exclusively to the water-soluble leaf components. There occur also relatively small losses of water-insoluble components. We mentioned on p. 160, as a minor part of the total weight losses, the removal of surface coatings consisting of waxes, resins, and embedded inorganic material. In a series of experiments, we determined by weighing the amounts of substances lost in this form by fermented tobacco samples, and found that they range between 0.8% and 2.1% of the BF dry weights, with an average value of 1.3%. It was further found that these surface coatings are, to a large extent, soluble in ethyl ether and insoluble in boiling water. This justifies allocating the loss of surface coatings to the water-insoluble fraction. This conclusion is supported by the independent analytical results that the ether-soluble solids of the leaf blades decrease during fermentation by about 1.5–2.5% of the BF dry weights. Although it is conceivable that this decrease is due to certain substances merely losing their solubility in ether without any appreciable loss of weight, it is nevertheless more probable that it corresponds, at least partly, to the removal of ether-soluble waxes, *etc.*, with the surface coatings. Further small weight losses of components of the insoluble fraction may originate from the demethylation of pectins accompanied by the liberation of methyl alcohol, and from residual decompositions of proteins, celluloses and hemicelluloses, involving the evolution of ammonia, carbon dioxide and water. However, losses of this latter kind can only be very small in view of the fact that the decomposition of proteins and high molecular carbohydrates have practically ceased, with the end of the previous stages of curing and of natural sweat. There are no data which would indicate weight losses of another nature imposed on the water-insoluble leaf components.

Altogether, the allocation of a weight loss of  $2.0 \pm 0.5\%$  (of BF dry weights) to the water-insoluble fraction appears to be a reasonable

estimate. This estimate is equivalent to an average loss of weight, to be attributed to the water-soluble components, of about 6.4% for the 1939 crop and of about 7.0% for the 1941 crop.

These figures were used for the evaluation of the amounts of soluble compounds which are, actually, converted into insoluble compounds, during the fermentation (Table III, No. 5). The higher value derived for the 1941 crop may be due to the fact that the tobacco of this crop, grown in an abnormally dry season, was particularly difficult to ferment, and had to undergo a considerably more drastic sweat process than the 1939 crop of tobacco.<sup>13</sup>

This shift of some of the leaf components into the water-insoluble fraction indicates that the fermentation is not a mere continuation of the curing process, the general trend of which is toward the breakdown of some of the leaf proteins into soluble nitrogen compounds, and toward the split of polysaccharides, pentosans, pectins, *etc.*, to lower molecular fragments of increased water solubility.

This reversal of the general trend of the reaction in the leaf tissues is, however, in all probability not equivalent to the renewed formation of the same types of higher molecular compounds which were solubilized in the previous stages of the tobacco processing. It has, rather, to be assumed that the newly formed compounds are chemically different from the water-insoluble components of the freshly harvested leaf.

Almost without exception, every individual tobacco sample analyzed by us shows, after fermentation, the shift of water-soluble leaf components into the water-insoluble fraction, the amounts of solids involved in this shift ranging from 1.5% to 5.5% of the BF dry weights. Generally, for every single sample, the shifted amounts increase with the effectiveness of its fermentation.

#### CHEMICAL NATURE OF THE SUBSTANCES SHIFTED INTO THE WATER-INSOLUBLE FRACTION

Several quantitative data and certain qualitative observations permit some conclusions as to the chemical nature of the leaf components which are subjected to the insolubilization effect.

<sup>13</sup> See also, in Fig. 1, the indication for a more extensive migration of potassium from midribs into blades, in the tobacco of the 1941 crop, and the larger effects for the 1941 crop shown in Table V.

*Inorganic Compounds*

The substances which change from a water-soluble to a water-insoluble form during the fermentation are not all organic compounds. Systematic analyses of the water-soluble inorganic components were carried out by evaporating and ashing water extracts prepared from every sample of a series, before and after its fermentation. Iron showed practically no change of its distribution between the water-soluble and the insoluble fraction, before and after fermentation. Calcium, phosphorus and manganese, however, were found in larger amounts in the water-insoluble fraction after the fermentation.

In Table IV, some of these values are presented, together with a calculation of the amounts which have become water-insoluble after the fermentation.<sup>14</sup> As the absolute amounts of manganese present in the leaves are smaller than those of calcium and phosphorus by one order of magnitude, we shall consider in the following discussion merely the changes of calcium and phosphorus.

There can be little doubt that some part of the shift of calcium is due to the decrease of the water solubility which calcium phosphates show at even slight increases of pH. On an average, the pH of tobacco extracts increases from about 6.5 to 6.9 in the course of the fermentation. The figures of Table IV for the shift of  $P_2O_5$ , if expressed as the corresponding amounts of calcium phosphates, show that, in both the 1939 crop and in the 1941 crop, approximately 0.07% of calcium<sup>15</sup> combine with the 0.10–0.11%  $P_2O_5$ , to form insoluble calcium phosphate during the fermentation. A further part of calcium may be insolubilized in the form of newly formed oxalate. The average increase of oxalic acid for the 1939 crop amounted to 0.18%, and for the 1941 crop, to 0.30%. This corresponds to the allocation of 0.08% calcium and of 0.13% calcium respectively to calcium oxalate formation.

This signifies that 0.35% of insolubilized calcium are still unaccounted for in the 1939 crop, and 0.57% calcium in the 1941 crop. It is probable that these quantities are combined with substances of the water-insoluble fraction, such as, *e.g.*, pectic acids, humic acids, *etc.*

We derive, in this way, a total of inorganic materials losing their water solubility during fermentation of 0.79–0.93% for the 1939 crop, and of 1.17–1.40% for the 1941 crop. The lower value of each pair is obtained by adding calcium phosphates, calcium oxalates, and residual

<sup>14</sup> The lost amounts of Ca,  $P_2O_5$  and Mn were based (1) on the difference between weight loss of leaf blades and apparent increases of Ca,  $P_2O_5$  and Mn, (2) on the content of Ca,  $P_2O_5$  and Mn in the removed surface gums. The losses are exclusively allocated to the insoluble fraction of the three constituents.

<sup>15</sup> All percentages mentioned here and in the following text are based on BF-dry weight, unless otherwise indicated.

TABLE IV

*Amounts of Ca, P<sub>2</sub>O<sub>5</sub> and Mn in Insoluble Fraction, Before (BF)  
and After Fermentation (AF)*

Pennsylvania seedleaf tobacco, leaf blades, averages of 30 representative samples per crop.

No.	Stage of processing	Amounts found expressed as	1939 crop			1941 crop		
			Ca	P <sub>2</sub> O <sub>5</sub>	Mn	Ca	P <sub>2</sub> O <sub>5</sub>	Mn
1	BF	Per cent of BF dry weight	2.75	0.14	0.010	2.27	0.15	0.008
2	AF	Per cent of AF dry weight	3.55	0.25	0.014	3.22	0.21	0.013
3	AF	Per cent of BF dry weight	3.25	0.21	0.013	2.93	0.20	0.012
4	AF	Per cent of BF dry weight, corrected for losses	3.28	0.25	0.013	3.04	0.25	0.012
5	Values No. 4 minus Values No. 1, or actual amounts made insoluble during fermentation.		0.53	0.11	0.003	0.77	0.10	0.004

calcium (as such), the higher value is obtained by adding calcium phosphates, calcium oxalates and the residual calcium converted to calcium oxide.

### *Organic Compounds*

By subtracting the amounts of insolubilized inorganic substances from the total solids shifted (Table III, No. 5), we conclude that the amounts of *organic* substances involved in the shift lie between 1.97% and 2.11% (1939 crop) and between 2.90% and 3.13% (1941 crop).

Certain analytical data throw some light on the nature of the organic substances which are insolubilized during the fermentation.

Parallel to the shift of organic substances into the water-insoluble fraction, a systematic *increase of insoluble*, or "*protein nitrogen*," goes hand in hand with the fermentation of every tobacco sample. The

term protein nitrogen has been employed by various authors for the total nitrogen found by Kjeldahl digestion in the residues of water-extracted tobacco samples.

Although there is little doubt that a major part of the water-insoluble nitrogen compounds contained in green and in cured tobacco leaves are, in fact, protein,<sup>18</sup> it would be erroneous, on the other hand, to conclude that the additional water-insoluble nitrogen compounds formed during fermentation are, necessarily, proteins, too.

TABLE V

*Amounts of Water-Soluble and Water-Insoluble Nitrogen, Before (BF)  
and After (AF) Fermentation*

Averages of 30 representative samples per crop.

No.	Stage of processing	Amounts found expressed as	1936 crop		1937 crop		1939 crop		1941 crop	
			Sol. N	Insol. N	Sol. N	Insol. N	Sol. N	Insol. N	Sol. N	Insol. N
1	BF	<i>Per cent</i> of BF dry weights	2.24 <sub>7</sub>	1.55 <sub>2</sub>	1.98 <sub>0</sub>	1.93 <sub>0</sub>	2.45 <sub>0</sub>	2.10 <sub>0</sub>	2.44 <sub>9</sub>	1.68 <sub>8</sub>
2	AF	<i>Per cent</i> of AF dry weights	1.93 <sub>6</sub>	1.89 <sub>4</sub>	1.65 <sub>0</sub>	2.26 <sub>0</sub>	2.02 <sub>1</sub>	2.42 <sub>9</sub>	1.94 <sub>0</sub>	2.19 <sub>0</sub>
3	AF	<i>Per cent</i> of BF dry weights	1.73 <sub>1</sub>	1.69 <sub>2</sub>	1.50 <sub>2</sub>	2.04 <sub>9</sub>	1.85 <sub>1</sub>	2.22 <sub>1</sub>	1.76 <sub>5</sub>	1.99 <sub>7</sub>
4	AF	<i>Per cent</i> of BF dry weights, corrected for losses of N	2.10 <sub>8</sub>	1.69 <sub>2</sub>	1.86 <sub>1</sub>	2.04 <sub>9</sub>	2.32 <sub>9</sub>	2.22 <sub>1</sub>	2.13 <sub>7</sub>	1.99 <sub>7</sub>
5	Values No. 1 minus Values No. 4. Actual conversion of soluble to insoluble nitrogen in <i>per cent</i> of BF dry weights.		.13 <sub>9</sub>		.11 <sub>9</sub>		.12 <sub>1</sub>		.31 <sub>2</sub>	
6	Insolubilized nitrogen-compounds (Values No. 5 multiplied by 6), in <i>per cent</i> of BF dry weights.		.83 <sub>4</sub>		.71 <sub>4</sub>		.72 <sub>8</sub>		1.87 <sub>2</sub>	

<sup>18</sup> See, e.g., Ref. (6). These authors proved that the nitrogen of the water-insoluble fraction of tobacco leaves is, in fact, almost exclusively protein nitrogen.

In Table V the averages of the soluble and the insoluble nitrogen are listed for various crops. The AF values, converted to the BF basis, and corrected for losses of total nitrogen are also contained in this table, together with the calculated amounts of insolubilized nitrogen, and of insolubilized nitrogen compounds. A loss of total nitrogen occurs with every successfully fermented sample, and, as a rule, amounts to roughly 0.2–0.4% of the BF weight, or to 5–10% of the total BF nitrogen. The average nitrogen losses per crop were allocated, in the calculations carried out for Table V, exclusively to the water-soluble nitrogen compounds. As already pointed out, any deamination of proteins or other insoluble nitrogen compounds during the fermentation appears negligible compared with the nitrogen losses incurred by the water-soluble nitrogenous components of the leaf tissues.

The values (No. 6, Table V) of the insolubilized nitrogenous compounds in the 1939 and 1941 crops represent a considerable fraction of the total insolubilized organic compounds (see p. 171) in these two crops. Subtraction of the former from the latter yields, for the 1939 crop, 1.24–1.38% of the BF dry weight, and for the 1941 crop, 1.03–1.26% of the BF dry weight. Evidently, these residual amounts of insolubilized organic compounds correspond to *non-nitrogenous* substances which accompany the inorganic and the nitrogenous components on their way into the water-insoluble fraction.

As to the nature of these residual organic substances only some qualitative observations are at present available. If 1 g. of a powdered, unfermented tobacco sample is extracted with 35 cc. of boiling water (reflux one hour), the color of the extract is appreciably darker than that of an extract, made under the same conditions, of the same tobacco sample after its fermentation. On the other hand, the water-insoluble residue of the extraction looks considerably darker for the fermented than for the unfermented sample. This indicates that the unknown insolubilized organic compounds are of a brownish color. It is well known that the brown coloration of air-cured tobacco is mainly due to the presence of polyphenols and of their oxidation and condensation products, commonly called tannins.<sup>17</sup> The tannins form with proteins, as well as with many lower molecular nitrogenous compounds, highly condensed products, which, as a rule, are insoluble in water. During the fermentation of tea leaves, which contain much larger amounts of tannins than tobacco leaves, a considerable portion of the tannins becomes water-insoluble, as outlined by E. A. H. Roberts (7).

These facts point to the possibility that tobacco tannins, during the fermentation, form complex nitrogen-containing products with some of the water-soluble nitrogenous components of the leaf tissue. These products appear in the "protein fraction"

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<sup>17</sup> See Ref. (1), p. 344 ff.



TABLE VI

*Protein Nitrogen Found After Extraction of Tobacco Sample H12, Crop 1941, with Various Extractants, and Increase of Insoluble Nitrogen, in per cent of BF Dry Weight, by Extraction of the Same Sample, After Fermentation (RH-12) with the Same Extractants*

No.	Solutions and solvents used for extraction	Nitrogen (Protein-N) left in extraction residue of BF samples, in per cent of BF dry weights	Increase of insoluble nitrogen in AF sample, on BF dry weight basis
1	Water containing 1.5% CuSO <sub>4</sub> and 0.3% NaOH (Barnstein method)	2.09 <sub>0</sub>	0.64 <sub>1</sub>
2	0.5% acetic acid (Mohr method)	1.84 <sub>7</sub>	0.51 <sub>3</sub>
3	Water	1.74 <sub>3</sub>	0.53 <sub>7</sub>
4	N/200 H <sub>2</sub> SO <sub>4</sub>	1.73 <sub>4</sub>	0.52 <sub>3</sub>
5	Water, no previous drying of sample at 65° C.	1.70 <sub>6</sub>	0.48 <sub>3</sub>
6	Water, followed by 75% alcohol	1.59 <sub>8</sub>	0.54 <sub>2</sub>
7	75% alcohol followed by water	1.54 <sub>8</sub>	0.51 <sub>1</sub>
8	3% aqueous NaHCO <sub>3</sub> solution	0.538	0.45 <sub>7</sub>

of the analysis. If this is so, this "pseudoprotein" will probably not remain associated in proportional amounts with the actual tobacco proteins, if the tobacco is subjected to extractions with various kinds of aqueous solutions or solvents which attack the proteins in various degrees. Table VI contains a survey of an experiment of this kind, carried out with tobacco powder of one and the same sample of the 1941 crop. The insoluble nitrogen, both for the unfermented and for the fermented sample, was determined in the residues which were left after extraction with the various solvents, some of which are being used in the determination of protein nitrogen. The extractions No. 1-No. 7 of the unfermented sample lead to protein nitrogen values ranging from 2.091% to 1.548% of the BF dry weight. The treatment with an aqueous 3% NaHCO<sub>3</sub> solution (No. 8) solubilizes considerable amounts of the protein nitrogen. In contrast to their different action on the tobacco proteins, all the eight extractants, affect only to a very small extent, if at all, the accrual of the insoluble nitrogen during the

fermentation. The values found for the newly insolubilized nitrogen for all the extractants, including the protein-dissolving sodium bicarbonate solution, average  $0.526 \pm 0.035\%$  of the BF dry weight. Obviously, the newly formed water-insoluble nitrogenous substances are considerably more stable against solvents than the original tobacco proteins, a property which is not in contradiction to what can be expected from complexes formed of tannins and nitrogenous compounds. It may even be possible to separate by complete solubilization of the protein proper, the product formed during fermentation. A decision as to whether polyphenols actually play an important role in the insolubilization effect during tobacco fermentation will have to be postponed until dependable analytical methods have been developed for the determination of the tannins of the tobacco leaf tissues.

Even without taking these observations into account, the formation of real proteins during the fermentation appears unlikely. It is highly improbable that a protein synthesis from soluble nitrogen compounds is effected in the tissues of the dead leaves. A protein formation may be ascribed to bacteria which are mostly found on fermenting tobacco leaves. A rough quantitative estimate, however, shows that the total protein nitrogen of the microorganisms found on tobacco leaves amounts, at best, to merely a fraction of the amounts found for the insolubilized nitrogen.

The microorganisms found by J. J. Reid, D. W. McKinstry and D. E. Haley (8) on well-fermenting Pennsylvania tobacco consisted mainly of *Micrococcus nicotianae* and *Bacillus subtilis*, with smaller numbers of *Bacillus megatherium*. The weights of these microorganisms are, in first approximation,  $0.5 \times 10^{-12}$  g.,  $0.8 \times 10^{-12}$  g. and  $1.8 \times 10^{-12}$  g. per single organism (9). The number of microorganisms/g. of tobacco change, according to Reid *et al.*, with the progress of the fermentation. The average numbers are  $10^7$  organisms, and the maximal number  $10^8$  organisms/g. of tobacco. This leads to total weights of microorganisms/g. of tobacco, between  $10^{-5}$  and  $10^{-3}$  g. The nitrogen content of *B. subtilis* and *B. megatherium* is 6-8% of the dry weight or about 1.2-1.6% of the total weight (10). This leads to only 0.000012-0.0016% (maximum) of microorganism nitrogen, of the tobacco weight, or to much lower values than those of the insolubilized nitrogen found after fermentation. Whether the protein nitrogen of dead microorganisms is accumulating, is a question which would still have to be decided.

Furthermore, a few preliminary experiments with aqueous extracts of unfermented tobacco samples which were incubated for 48 hours at 50°C. under sterile conditions (previous boiling, presence of toluene) show that, even in the absence of microorganisms, water-insoluble precipitates are formed which contain considerable amounts of nitrogen. If further experiments of this type should prove that some of the typical reactions of the fermenting leaves can be duplicated with

incubated sterilized leaf extracts, they may contribute useful information on the chemical mechanisms of the cigar tobacco fermentation.

## EXPERIMENTAL PART

### *Fermentation of Small Leaf Samples in Closed Containers*

Eight tin-plated steel tubes (internal diameter 4 cm., length 50 cm.) served as containers for about 15 leaves each. To effect a tight seal, the open ends of these tubes could be closed by soldering over them overlapping, tin-coated steel caps. The latter carried, centrally, a tube of 6 mm. internal diameter to permit the inlet and outlet of gases into the main (tobacco) tubes. By rubber hose and glass tube connections, the inlet tubes were connected with a humidifier, consisting of a wide vertical glass cylinder, filled with porcelain rings over which water trickled slowly from top to bottom. Behind the humidifier, the system was connected with either a small air blower, or with an oxygen, hydrogen or nitrogen flask. The gas, after passing the humidifier and the tobacco tubes, left the system over a series of wash bottles filled with dilute acid and dilute alkali solutions, for the absorption and titration of volatile bases and carbon dioxide generated by the tobacco leaves. Simple mercury manometers served for measuring the pressure in each tobacco tube; stopcocks permitted isolating every tobacco tube and its manometer from the remainder of the system. The eight tobacco tubes were immersed in a large thermostat. In direct contact with the latter, a second smaller thermostat contained the humidifier. The tubes were filled with 15 leaves each of tobacco which was ready for fermentation (cured, finished with its natural sweat), closed by sealing the caps, connected with the gas inlets and outlets, and immersed in the thermostat which was usually kept at 45°C. The temperature of the humidifier thermostat was regulated so that the moisture of the gases, after passing the humidifier, amounted to an optional value between 30% and 70% relative humidity, at 45°C.

The inlets and outlets to the tobacco tubes remained closed for most of the time during which fermentation of the leaves was expected to take place. At the same intervals (*e.g.*, in succession, after one day, two more days, four more days, six more days) at which the same sample of tobacco had to be aired, if fermented in the usual way, humidified gas was passed over the leaves in the steel tubes. Preliminary tests had shown that a slow gas stream applied for 5 minutes provided a sufficient gas exchange. Any longer treatment produced no other effect than a too rapid drying of the leaves. The gas which passed over the leaves, during this 5 minutes' treatment, was analyzed for volatile bases and carbon dioxide. After each gas treatment, the tobacco containers were again closed.

If treated with air or oxygen, the leaves showed, in most of the experiments, the characteristic changes of fermentation, although they did not sweat as satisfactorily as did tobacco of the same type which was fermented in the usual way. Appreciable amounts of organic bases, particularly ammonia, and of carbon dioxide were generated. After each air or oxygen treatment, the pressure in the sealed tobacco containers had decreased by several mm. of Hg during the time interval of one gas treatment to the next. The same procedure was carried out with other leaves of the same tobacco sample, using hydrogen, and in another series with nitrogen instead of air

or oxygen. No visible signs of any fermentation could be detected in these runs. The odor of the leaves, after such a treatment, had changed to an undesirable smell. The hydrogen and nitrogen, after passing the tobacco tubes, contained smaller amounts of volatile bases and of carbon dioxide than the air or oxygen under the same condition, and these amounts decreased with repeated hydrogen and nitrogen treatments of the leaves faster than they decreased in the air and oxygen experiments. No pressure decrease in the tobacco tubes was observed in the course of the hydrogen or nitrogen experiments.

### *Direct Determination of Weight Losses*

During its industrial processing through the natural sweat and the fermentation, the tobacco is handled in units of "cases," each of which contains 250-300 pounds of tobacco. The tobacco is packed in these wooden cases in the form of individually tied bundles of 15-20 entire leaves, the so-called "hands"; the hands are arranged in regular rows within the cases. The cases which served as a source of our analytical samples ("experimental cases") were chosen in such a way as to represent a cross section of an individual crop (various sizes, origins, qualities). Before the tobacco of an experimental case was "cased" prior to its fermentation (*i.e.*, dipped in water to take up an individually selected amount of additional moisture), 10 hands were taken, in a random sampling procedure, from this case (BF samples). Thereafter, the weight of the remaining bulk of tobacco was determined. Two leaves of every one of the withdrawn 10 hands were immediately weighed, dried for 2 hours at 124°C. in a "Freas" oven (thermostatically controlled, mechanical convection), and reweighed. The moisture content of the BF tobacco derived from this determination was used to derive the initial dry weight of the bulk tobacco before its fermentation. During the entire fermentation, the tobacco was carefully handled to keep mechanical losses at a minimum. All leaf fragments and scrap tobacco which came off during the airing and other operations were collected, weighed and listed for every experimental case. At the end of the fermentation, the bulk of tobacco was weighed again, 10 hands taken as AF samples, and again 2 leaves/hand used for moisture determinations.

The total loss of solids during the fermentation was evaluated as the difference of the BF dry weight of the bulk tobacco, and its AF dry weight, the latter being corrected for the relatively small breakage loss.

### *Analytical Determinations*

Normally, the chemical analysis of the tobacco samples was made for the leaf blades only. The leaves contained in the 10 sample hands withdrawn for analysis (see previous paragraph) were "stripped," by removing the midribs of every single leaf. The midribs were discarded except in those experiments in which separate analyses of the midribs were carried out. The remaining leaf-blades were spread and air-dried at room temperature. After about 20 hours, the blades had become very brittle, their moisture content being 5-8%. In this state, they were ground in an attrition mill to a tobacco powder of an average size of 40-70 mesh. The powders were stored in glass containers with tightly fitting screw caps at refrigerator temperatures. Repeated analytical checks made after storage periods of several years proved that, under these

conditions, the chemical composition of the tobacco powder undergoes no detectable change. All the subsamples used for analytical determinations were dried for one-half hour in the constant-temperature oven in an air-stream at 68°C. and weighed. These weights were used as the basis of "dry weight" for expressing the analytical results.

*Inorganic Components.* The tobacco powder was ashed in a muffle furnace, at about 550°C. until no further signs of ignition could be detected. The ash was treated in 3 different ways: (a) for determination of Ca, Fe and P; (b) for determination of K; and (c) for determination of Mn.

(a) The ash is treated with concentrated boiling hydrochloric acid until  $\text{CO}_2$  evolution has subsided. The solution plus insoluble parts is evaporated to dryness, and the residue treated with a boiling mixture of 1 part of concentrated hydrochloric acid and 4 parts of water, for 1/2 hour. The undissolved residue is filtered off, and the solution made up to a given volume (Soln. 1). After ignition on the filter paper, the residue is treated with a boiling mixture of 1 part of concentrated  $\text{H}_2\text{SO}_4$  and 1 part of water, until all the liquid has been evaporated. The residue is taken up with hydrochloric acid (1 + 4), filtered, and made up to the same volume as Soln. 1 (Soln. 2). The still insoluble portion represents silica; it is ignited on the filter paper and weighed. Equal parts of Soln. 1 and Soln. 2 are used for the determination of Ca, P, and Fe. Ca is determined as the oxalate by the titration method with  $\text{KMnO}_4$  (11); Fe by the color reaction with phenanthroline (12), and P by the molybdenum blue colorimetric method.<sup>18</sup> These three methods were selected, after a thorough study of several other methods, as the most suitable and dependable ones for our special problem.

(b) For the determination of K, another aliquot of the ash is treated with a boiling hydrochloric acid-water mixture (1:4) which is evaporated to dryness. The residue is taken up with a dilute hydrochloric acid-water mixture (about 1:50), and made up to a given volume. Potassium is precipitated as the chloroplatinate which is filtered off and washed.<sup>19</sup> The washed precipitate is dissolved in water, 4 cc. conc.  $\text{HCl}$  are added/100 cc. of this solution, and about 0.5 g. of metallic magnesium powder for every 0.2 g. K present, to precipitate Pt in metallic form. After repeated filtering and washing, the platinum is ignited and weighed. 0.4006 times the weight of Pt found is equal to the K contained in the aliquot.

(c) For the determination of Mn, a nitric acid solution of the ash is required. Concentrated  $\text{HNO}_3$  is added to the ash, and the mixture is boiled for 30 minutes, filtered, and the filtrate made up with about 2 volumes of water to a given volume. To an aliquot of this extract, 3-5 cc. of concentrated phosphoric acid, and about 0.3 g. potassium periodate are added, in successive small portions. The solution is boiled for a few minutes until the color of permanganate which develops if Mn was contained in the ash, does not increase any further. The amount of manganese in the aliquot is determined colorimetrically against a  $\text{KMnO}_4$  standard of known concentration.

*Water Extractions of Tobacco Powders, Determination of Water-Insoluble Solids.* For the determination of the water-soluble inorganic components, a given dried

<sup>18</sup> See Ref. (11), p. 133.

<sup>19</sup> See Ref. (11), p. 129.

amount of tobacco powder is extracted with boiling distilled water (35 cc. for 1 g. of tobacco powder) under the reflux condenser, for one hour. The residues are filtered off and washed with hot water so that the filtrate comes to a volume of about 60 cc. for 1 g. of tobacco powder. This filtrate is slowly evaporated to dryness on the steam bath, and ashed. The analysis of this ash is carried out in the same way as that of the total ash of the original tobacco powder.

The same extraction method was used for the determination of the water-insoluble solids. No differences were found if 1/200  $N$   $H_2SO_4$  was used, instead of water, as an extractant. The residues of these extractions were air-dried, and then weighed, after drying in the oven at 65°C. for one-half hour.

Checks of these values by determinations of the water-soluble solids were satisfactory.

*Nitrogen Determinations.* The nitrogen determinations discussed in this paper<sup>20</sup> are all based on Kjeldahl analyses. After various preliminary studies, it was finally established that the total nitrogen in tobacco powders and in water-extracted tobacco powders<sup>21</sup> is fully recovered by Kjeldahl analyses if the digestion is carried out in presence of selenium as a catalyst.<sup>22</sup>

The *total nitrogen* was determined by the direct digestion of dried powder samples. The *water-insoluble* ("protein") nitrogen was determined in residues of water-extracted powder samples. The extractions were carried out as described above, except that, as a safety measure, the water was replaced by 1/200  $N$   $H_2SO_4$  (to avoid losses of volatile nitrogenous bases). Comparative experiments showed that both the weights of the water-insoluble solids as well as the amounts of water-insoluble nitrogen were the same, whether a given sample was extracted with distilled water or 1/200  $N$  acid.

The Kjeldahlization included the following steps: *Digestion* of the sample with 30 cc. of sulfosalicylic acid (1 g. salicylic acid in 30 cc. conc.  $H_2SO_4$ ). The mixture stood for 12 hours at room temperature. Then, 5 g. of sodium thiosulfate are added (dry), and the mixture kept at room temperature for another 4 hours. Thereafter, the mixture is brought to boiling. As soon as white fumes appear, 3 "Hengar Granules, Selenized" (Hengar Co., Philadelphia) are added. After one further hour of boiling, one more granule is added to the mixture. Altogether, the digestion with the boiling mixture is continued for 16 hours, although the liquid appears colorless at a much earlier time. After cooling, addition of water, and renewed cooling, the solution is made alkaline, and distilled into 1/20  $N$   $H_2SO_4$ . The excess of acid is back titrated with 1/2  $N$   $NaOH$ , using methyl red as indicator. The (very small) nitrogen content of the reagents used was determined in a series of blank experiments, and used to correct the titration values.

<sup>20</sup> The results of detailed analyses of nitrogenous components of the leaves will be described in a subsequent paper.

<sup>21</sup> The total nitrogen of aqueous extracts of tobacco cannot be determined by mere Kjeldahl digestions. These extracts require first the removal and determination of all the nitrate nitrogen, followed by Kjeldahl determination of the residual nitrogen.

<sup>22</sup> Neither mercury nor copper-salts proved to be efficient enough catalysts for the Kjeldahl digestion of the heterocyclic nitrogen compounds contained in tobacco leaves.

## ACKNOWLEDGMENTS

The numerous analyses and other systematic experiments were carried out by Mrs. Marion Bender-Kutz, Mr. Fuad Daud, Dr. Pauline Kulka, Miss Marlaese Metzger and Miss Phyllis Tweed in this laboratory.

The fermentation of the experimental samples were supervised and commented on by Mr. J. Strauss of the Resweat Plant in Lancaster.

The author wishes to express his appreciation to Messrs. B. G. Meyer and I. B. Wolfner, General Cigar Co., New York, who made this work possible.

## SUMMARY

The fermentation of Pennsylvania cigar leaf tobacco proceeds in an air or oxygen atmosphere but not in an hydrogen or nitrogen atmosphere. The leaves of well-fermented tobacco lose 7-10% of total solids during the fermentation. The main part of this loss is caused by the evolution of  $\text{CO}_2$ ,  $\text{NH}_3$  and probably of  $\text{H}_2\text{O}$ , as products of oxidative reactions within the leaf tissues; a smaller part of the loss originates from the removal of gummy surface layers of the leaves. The apparent increase of the calcium and potassium contents of the leaves check, in first approximation, with the decrease of the total tobacco weight. Parts of the Fe,  $\text{P}_2\text{O}_5$  and  $\text{SiO}_2$  contained in the leaves are lost, probably by inclusion in the removed surface layers.

During the fermentation, water-soluble components of the leaves, amounting to 2-4% of the tobacco weight, become water-insoluble. This effect is due to (1) the formation of calcium phosphate, calcium oxalate and other water-insoluble calcium compounds, (2) the shift of some of the water-soluble nitrogen compounds into the water-insoluble ("protein-nitrogen") fraction, and (3) the insolubilization of other unidentified non-nitrogenous organic leaf components.

Several quantitative data and qualitative observations point to the possibility that changes (2) and (3) are caused by the formation of water-insoluble complexes between polyphenols and nitrogenous compounds within the leaves.

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# Metabolism of $\beta,\delta$ -Diketohexanoic Acid in Minced Tissues<sup>1</sup>

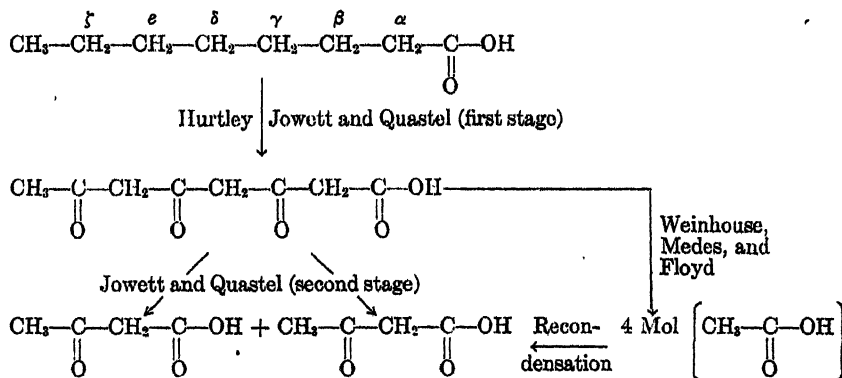
F. L. Breusch and E. Ulusoy

*From the Second Institute of Chemistry, University of Istanbul, Turkey*

Received April 4, 1947

## INTRODUCTION

In addition to the theory of simple  $\beta$ -oxidation of Knoop (1904), Hurtley (1916) and, more clearly, Jowett and Quastel (1935) proposed a theory of alternating  $\beta$ -oxidation, consisting of two parts. According to their opinion the first step of biological attack in the breakdown of fatty acids consists not only in the introduction of oxygen at the  $\beta$ -carbon atom, but also at every succeeding second C-Atom. In a second reaction this  $\beta, \delta, \zeta$ -oxygenated fatty acid chain will break down by pure hydrolysis, forming as many molecules of acetoacetic acid as can be furnished by the number of C-atoms in the chain divided by 4. The theory is based on the fact that octanoic acid with liver slices forms more than one molecule of acetoacetic acid. According to the theory of Knoop, only one molecule of acetoacetic acid was accounted for. Experiments of Lehninger (1945) confirmed the experimental results of Jowett and Quastel.



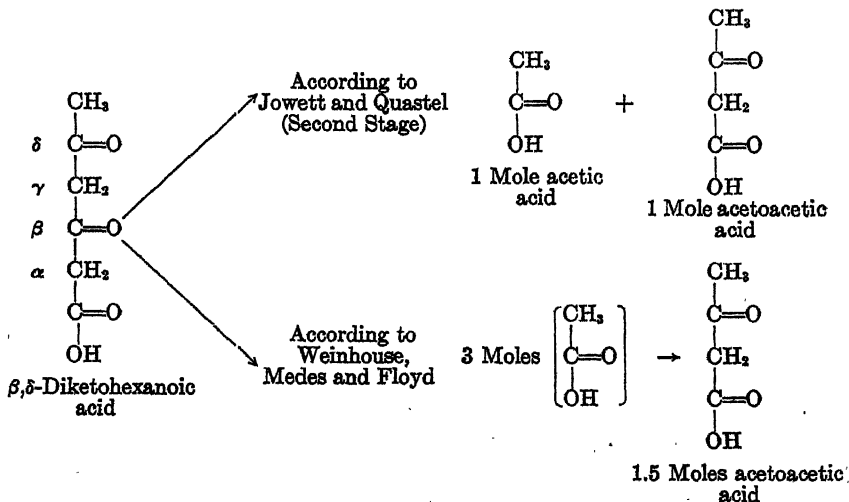
<sup>1</sup> Dedicated to Professor Carl Neuberg on his 70th birthday.

Later Weinhouse, Medes and Floyd (1944) found that octanoic acid, labeled in the carboxyl group with isotopic  $C^{13}$ , produced acetoacetic acid, containing  $C^{13}$  not only in the carboxyl group, but also in the  $\beta$ -carbonyl group. This was evidence that the second part of the Jowett-Quastel theory, dealing with the formation of acetoacetic acid, really consists of two steps, first a breakdown of the whole chain to acetic acid or radical-like  $C_2$  parts, as proposed by Martius (1943), and their subsequent recondensation to acetoacetic acid. The possibility of enzymatic recondensation of acetic acid to acetoacetic acid by liver has been known for a long time.

Contrary to clearing up the second part, a direct decision on the reality of the first part of the Hurtley-Jowett-Quastel theory, alternating  $\beta$ -oxidation, seems difficult. The supposed intermediate product of the breakdown of octanoic acid in liver, the unknown  $\beta,\delta,\zeta$ -triketoocanoic acid, cannot be proved to be metabolized in the liver.

But the homologue,  $\beta,\delta$ -diketohexanoic acid (triacetic acid), is well known. If the theory of the alternating  $\beta$ -oxidation is correct, then  $\beta,\delta$ -diketohexanoic acid must also form acetoacetic acid. Hexanoic and octanoic acid show no great difference in their transformation to acetoacetic acid in liver.

If the  $\beta,\delta$ -diketohexanoic acid is metabolized according to the second step of the Jowett-Quastel concept, only one molecule of acetoacetic acid in addition to one molecule of acetic acid should be formed. If the acid is metabolized according to Weinhouse, Medes and Floyd, 1.5 molecules of acetoacetic acid should be formed:



Our results show definitely that  $\beta,\delta$ -diketohexanoic acid, incubated with liver suspensions, forms more than one molecule, usually about 1.3 molecules of acetoacetic acid, which has been analyzed and identified as acetone.

This result makes it probable that in liver the first reaction of the theory, alternating  $\beta$ -oxidation of the fatty acid chain, really occurs; and that the second reaction consists of the splitting up of the chain into  $C_2$  fragments. This is followed by a nearly quantitative recondensation to acetoacetic acid.

These investigations also explain the results of Dakin (1923), that liver perfusion with hexanoic acid,  $\beta$ -hydroxyhexanoic acid,  $\beta$ -ketohexanoic acid and  $\alpha,\beta$  unsaturated hexanoic acid yield only acetone, and no methylpropylketone, as was to be expected according to the theory of single  $\beta$ -oxidation.

All other organs, such as kidney, muscle, brain and lung, do not metabolize the  $\beta,\delta$ -diketohexanoic acid to a measurable extent. Within the limits of error of our analytical methods the acid remains unaltered by incubation with these tissues. In these tissues the main metabolism of the fatty acids can not, therefore, pass through the mechanism of alternating  $\beta$ -oxidation. With these the concept of simple  $\beta$ -oxidation of Knoop and the subsequent breakdown of the first two C-atoms *via* the tricarboxy acid cycle, according to the theory of Breusch (1943), remains valid. Contrary to the behavior of other  $\beta$ -keto acids in muscle the  $\beta,\delta$ -diketohexanoic acid does not enzymatically combine with oxaloacetic acid to form citric acid (Breusch and Keskin, 1944). According to Breusch and Tulus (1945) free fatty acids, incubated with oxaloacetic acid in fresh suspensions of pigeon muscle, yield considerable amounts of citric acid. Since  $\beta,\delta$ -diketohexanoic acid is unable to do so, this acid can not be considered as an intermediate substance in the fatty acid breakdown in muscle, as it probably is in liver. Fundamental differences between different tissues in the metabolism of fatty acids, especially the ability to form acetoacetic acid, have already been mentioned by Snapper, Grünbaum and Neuberg (1926). Liver also contains little of the enzyme system capable of combining  $\beta$ -keto fatty acids with oxaloacetic acid, which kidney and muscle possess in abundance (Breusch, 1943, 1944).

## EXPERIMENTAL

*Methods*

Crystalline  $\beta,\delta$ -diketohexanoic acid (triacetic acid) was prepared as the enol-lactone according to Collie (1891). Cats, starved for 4 days, were used. These were killed by a blow on the head, the organs immediately removed, minced in a Latapie mincer and suspended in a 0.9% NaCl solution. Addition of phosphate changed nothing. After adding a neutralized solution of the acid, it was shaken for 40–60 minutes at 38°C. in 250 ml. Erlenmeyer-flasks.

*Analytical*

For the estimation of *acetone* the suspensions were deproteinized with  $\text{Na}_2\text{WO}_4$  and  $\text{H}_2\text{SO}_4$ . The whole mixture was then distilled from an all glass distillation apparatus without filtration. The distillate, 25 ml., was directly distilled into the main vessel of a second all glass distillation apparatus, from which 10 ml., containing all the acetone, were distilled to a graduated cylinder. Of this, an aliquot was diluted with water to 4 ml.; 0.3 ml. of salicylaldehyde and 2 ml. of conc.  $\text{H}_2\text{SO}_4$  were added. The whole mixture was vigorously shaken and heated for 15 minutes in a boiling water bath. After cooling, 5 ml. of ethanol were added and the red color was compared with a blank in a Zeiss Stufenphotometer using filter S 53 and a 5 mm. cuvette. Acetone was also identified as the crystallized 2, 4-dinitrophenylhydrazone.

$\beta,\delta$ -Diketohexanoic acid decomposes under our conditions of distillation to about 5% of its weight, forming acetylacetone. This  $\beta,\delta$ -diketoacid is surprisingly stable on boiling with dilute acids, as it is present in the form of a stable internal enol-lactone. No trace of acetone is formed. The acetylacetone formed gives per mg. about the same color with salicylaldehyde as acetone, but differs from acetone in giving only a very small precipitate with a 0.5% solution of dinitrophenylhydrazine in 2 *N* HCl. The corrections necessary for the formation of acetylacetone, are subtracted; they are small, between 1 and 5% of the total "acetone" found.

The analysis of  $\beta,\delta$ -diketohexanoic acid was carried out according to the analytical method as developed by Pucher, Sherman and Vickery (1936) for citric acid. Breusch and Tulus (1947) have shown that this colorimetric method is not specific for citric acid, and that some other acids, which by oxidative bromination form bromoketones, yield the same color with  $\text{Na}_2\text{S}$  solution. For the estimation, aliquot amounts of the tissue suspensions were deproteinized with an equal amount of a 10% solution of trichloroacetic acid in water. From the clear filtrate an aliquot part was boiled for 2 minutes to remove the acetoacetic acid. After cooling,  $\text{H}_2\text{SO}_4$ , KBr, and  $\text{KMnO}_4$  solutions were added, as suggested by Pucher, Sherman and Vickery, but omitting the treatment with bromine water. Further treatment was according to the directions for citric acid. The color intensity produced by  $\beta,\delta$ -diketohexanoic acid is about 15% of the same molar amount of citric acid. As compared with the enormous sensitivity of the method for citric acid the color intensity for  $\beta,\delta$ -diketohexanoic acid was still great enough to determine 1 mg. of the diketoaicid in 100 ml. A differentiation from citric acid is not possible. But a conversion to citric acid is improbable, as muscle and kidney rapidly oxidize citric acid (Breusch and Tulus, 1946), while they do not oxidize  $\beta,\delta$ -diketohexanoic acid.

## RESULTS

TABLE I

*Disappearance of  $\beta,\delta$ -Diketohexanoic Acid in Different Tissues*

15 g. of minced tissue + 100 mg. of neutralized  $\beta,\delta$ -diketohexanoic acid lactone in 35 ml. of 0.9% NaCl solution.

Organ	$\beta,\delta$ -Diketohexanoic acid			"Ketone" formed	Metabolizing capacity/100 g. wet tissue/hour
	Immediately	30 min.	60 min.		
Liver, cat	mg. 95	mg. 40	mg. 8	mg. 80	mg. 580
Kidney, cat	89	87	86	9	20 (?)
Brain, cat	92	89	90	5	13 (?)
Lung, cat	97	95	95	2	13 (?)
Muscle, pigeon	97	95	95	7	13 (?)

Six hours after death, at room temperature, the ability of the liver to metabolize  $\beta,\delta$ -diketohexanoic acid ceased. By grinding the minced tissue for 5 minutes with 3 times as much water at 0°C., followed by 10 min. centrifugation at 3000 r.p.m., most of the activity shifts to the colloidal supernatant liquid; 5 min. at 55°C. destroys any activity.

As recorded in the last column, acetone, which was added or formed, remains practically unchanged and does not seem to serve as a metabolite, within the limits of our methods. It is an interesting fact, that  $\alpha,\gamma$ -diketohexanoic acid (pure, crystalline), unlike  $\beta,\delta$ -diketohexanoic acid, produces no traces of excess acetone.

To decide the question of how much of the colorimetrically found "acetone" was really acetone, the following test was made: 60 and 45 g. of the liver of cats which had been previously starved for 4 days + 300 mg. of  $\beta,\delta$ -diketohexanoic acid enol lactone, neutralized in 90 ml. of 0.9% NaCl, was shaken for 60 min. at 38°C. After deproteinization, the remaining  $\beta,\delta$ -diketohexanoic acid was analyzed and the acetone distilled, as before. To the distillate (20 ml.) the acetone content of which had been colorimetrically analyzed previously, the necessary amount of 2,4-dinitrophenylhydrazine, as a clear 0.5% solution in 2 N HCl, was slowly added. Every addition made after the first-formed colloidal precipitation of acetonedinitrophenylhydrazone, crystallized. Under our conditions the hydrazone often remained colloidal, if the addition of the solution of dinitrophenylhydrazine was made too rapidly or if a

TABLE II

*Formation of Acetone from Different Hexanoic Acids in Liver*

15 g. of cat liver, 80 mg. of neutralized substance in 35 ml. of 0.9% NaCl.

Substance added	Acetone found after 60 minutes	Surplus acetone found; ex- pressed in Mole-% of the added substance
None	mg. 1.5	per cent control
$\beta,\delta$ -Diketohexanoic acid	59	159
Hexanoic acid	1.9	0.6
$\delta$ -Ketohexanoic acid	1.9	0.5
$\alpha,\gamma$ -Diketohexanoic acid	1.5	0
Acetone, pure	75	93

great excess was added. After standing for 48 hours at room temperature, the yellow-red needles were filtered off, washed with few ml. of cold 2 N HCl and water, dried and weighed. The melting point was 121–123°C. After one recrystallization from water it was 126–128°C. The substance gave no melting point depression with an authentic sample of acetonedinitrophenylhydrazone having a melting point of 127–128°C.

TABLE III

*Quantitative Isolation of Acetone, Formed from  $\beta,\delta$ -Diketohexanoic Acid*

1 Liver	2 Acid added	3 Unchanged acid remaining	4 Metabol. acid	5 "Acetone" colorim.	6 Acetone found by precipitation	7 Acetone isolated as % of acid disapp.
g. 60	mg. 300	mg. 70	mg. 230	mg. 228	mg. 135	per cent 129
45	300	134	166	164	98	128

In column 5 the acetylacetone formed by a 5% splitting of unaltered  $\beta,\delta$ -diketohexanoic acid, 3 or 5 mg., is subtracted.

In column 6 the amount of the acetonedinitrophenylhydrazone, which, because of the solubility of the hydrazone, 88 mg./100 ml. of 2 N HCl, is not precipitated, has been added.

In column 7 the formation of 1 mole of acetone (m.w. = 58) from 1 mole  $\beta,\delta$ -diketohexanoic acid enol lactone (m.w. = 126) is reckoned as a 100% yield.

By repeating the same experiments with livers of unstarved cats the percentage amount of acetone formed is smaller, sometimes only one-half. With starved cat liver, more than 100% of acetone was always found.

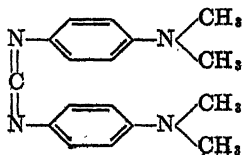
Similar experiments, in which the deproteinized filtrates were investigated for  $\beta$ -oxybutyric acid, possibly formed by reduction of the intermediate acetoacetic acid, showed no increase as compared with blanks. Also, the formation of acetoin showed no increase. As a possible intermediate between the breakdown of  $\beta,\delta$ -diketohexanoic acid and acetoacetic acid, acetic acid might have accumulated. But liver filtrates which had been incubated with  $\beta,\delta$ -diketohexanoic acid contained no acetic acid. These filtrates were extracted for one week with ether and the ether extract condensed with bis(*p*-dimethylaminophenyl)-carbodiimid, according to Breusch and Ulusoy (1946).<sup>2</sup> No ureid of acetic acid formed.

### DISCUSSION

As a result of these experiments, it seems probable, that alternating  $\beta$ -oxidation occurs only in the liver. Liver, as the central organ of metabolism, splits  $\beta,\delta$ -diketohexanoic acid to  $C_2$  fragments with immediate recondensation to acetoacetic acid. No measurable amount of acetic acid as a probable intermediate product could be detected. Contrary to this, in peripheral tissues, such as muscle and kidney, the  $\beta,\delta$ -diketohexanoic acid is either not at all or only to a small extent metabolizable; neither does it form acetoacetic acid, nor metabolize as a  $\beta$ -ketoacid *via* the tricarboxy acid cycle according to Breusch (1943). The  $\alpha,\gamma$ -oxidation of the fatty acid chain, as proposed by Witzemann (1942), and also by Jowett and Quastel, seems unlikely, as pure

<sup>2</sup> The reacting substance melting at  $86^\circ C.$ , as mentioned in the quoted paper, is not bis(*p*-dimethylaminophenyl)-urea, but has one molecule  $H_2O$  less, and is bis(*p*-dimethylaminophenyl)-carbodiimid.

The error is due to the fact that the publication of Zetzsche was only available to us as an abstract.





crystallized  $\alpha,\gamma$ -diketohexanoic acid, contrary to  $\beta,\delta$ -diketohexanoic acid, gave no trace of acetone, while hexanoic acid does.

Liver has but slight ability to condense acetoacetic acid and oxaloacetic acid to citric acid or another of the tricarboxylic acids. On the other hand, liver can easily form considerable amounts of acetoacetic acid by alternating  $\beta$ -oxidation. It seems likely that, in the intact organism, liver carries out the first step in breaking down fatty acids, while the second step, burning down acetoacetic acid, is mainly done in the peripheral tissues over the mill of the tricarboxy acid cycle, as discovered by Breusch (1943) and Wieland and Rosenthal (1943), denied by Krebs and Eggleston (1944), but definitely proven by Weinhouse, Medes and Floyd (1946). Independent of this, the peripheral organs, muscle and kidney, seem to be able to oxidize fatty acids according to the old concept of Knoop (1904), forming only  $\beta$ -hydroxy and  $\beta$ -keto acids, which are then metabolized *via* the tricarboxy acid cycle. The normal breakdown of fatty acids in peripheral tissues does not seem to proceed through the alternative  $\beta$ -oxidation, but only through simple  $\beta$ -oxidation.

In the kidney sometimes the presence of  $\beta,\delta$ -diketohexanoic acid induces formation of volatile "ketone," although the non-volatile  $\beta,\delta$ -diketohexanoic acid remains practically unchanged. To a small extent this phenomenon is also observed in muscle. The cause will be investigated further.

### SUMMARY

$\beta,\delta$ -Diketohexanoic acid (triacetic acid) is easily metabolized by liver (300–600 mg./hr./100 g. of wet tissue). Almost the quantitative amount of acetoacetic acid is formed. The acetone, isolated from acetoacetic acid, was identified as acetonedinitrophenylhydrazone having a m.p. of 128°C.

In kidney, muscle and lung the acid remains practically unattacked.

The results seem to render probable the Hurtley-Jowett-Quastel theory of alternating  $\beta$ -oxidation in liver. At the same time they exclude the validity of this concept in the breakdown of fatty acids in peripheral organs, such as kidney and muscle. For these the Knoop theory of simple  $\beta$ -oxidation followed by the breakdown over the tricarboxylic acid cycle still remains the most probable.

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# Studies in Chemotherapy of Tuberculosis IV<sup>1</sup>

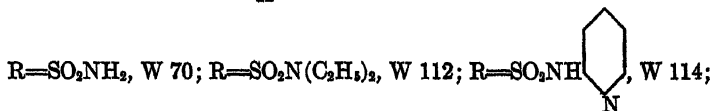
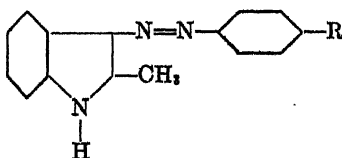
Harry Willstaedt and Marthe Borggård

*From the Wenner-Gren Institute of Experimental Biology,  
University of Stockholm*

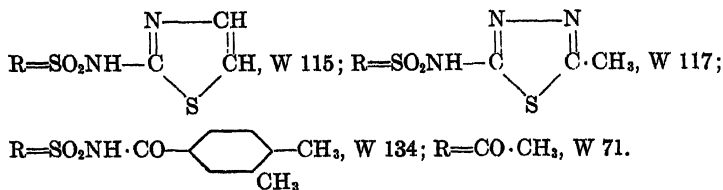
Received February 28, 1947

## INTRODUCTION

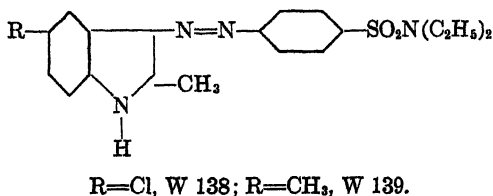
One of the various methods which may possibly produce chemotherapeutic compounds with a specific effect against tuberculosis is the introduction of a radicle structurally corresponding to one of the components of the tubercle bacillus into substances with a known chemotherapeutic effect. In several instances the preparation of fatty acid derivatives of sulfanilamides has been employed for this purpose. Our own experiments are, however, based on the findings of Anderson and Newman (1) that the tubercle bacillus contains phthiocol pigment (2-methyl-3-hydroxynaphthoquinone). In previous papers (2, 3) we described a number of substances, both azo dyes and triphenylmethane dyes, which contain 2-methylnaphthalene radicals. Instead of 2-methylnaphthalene the structurally related 2-, 6- and 7-methylquinolines were used in later experiments (4). The present paper is to describe the synthesis of some substances in which the 2-methylnaphthalene has been replaced by the isosteric 2-methylindole. In these compounds there is an azo linkage between the methylindole and various sulfanilamides or similar substances. They have the following general formula:



<sup>1</sup> Dedicated to Professor Carl Neuberg on his 70th birthday.



In order to ascertain the influence possibly exerted upon the chemotherapeutic activity by substituents in the methylindole ring, the following dyes were also synthesized:



Another synthesis was made of a substance (W 113) constitutionally corresponding to W 71 but not containing any methyl group whatsoever in the indole ring.

### EXPERIMENTAL

**Diazotization.** 0.01 mol of the diazo components (except N<sup>1</sup>-3,4-dimethylbenzoyl-sulfanilamide<sup>2</sup>) were dissolved in 20 ml. of water and 3 ml. of concentrated HCl. They were then diazotized in the usual manner with a solution of 0.7 g. of sodium nitrite in 5 ml. of water. The resulting diazonium salts were added to solutions of 1.3 g. (0.01 mol) of 2-methylindole and 5 g. of sodium acetate in 150 ml. of ethanol. After 24 hours the dyes were precipitated by dilution with water and were recrystallized from dilute ethanol.

### DISCUSSION

The object of the present research was, first, to produce, if possible, a substance with a marked chemotherapeutic activity and, second, to ascertain the influence exerted by certain substituents on the activity of a given molecule. The bacteriological tests of the substances in question were carried out by B. Zetterberg at the Institute of Hygiene and Bacteriology of the University of Uppsala. Culture experiments on the Löwenstein medium did not produce extraordinarily marked

<sup>2</sup> This compound was diazotized by adding dropwise an aqueous solution of 3.3 g. of its sodium salt and 0.7 g. of sodium nitrite to a mixture of 5 ml. of concentrated HCl and 20 ml. of water.

Sub- stance number	Formula	Molec- ular weight	Melting point	C		H		N	
			°C.	Calc.	Found	Calc.	Found	Calc.	Found
W 70	$C_{15}H_{14}O_2N_4S$	314	137-138	—	—	—	—	17.8	17.5
W 71	$C_{17}H_{15}ON_4$	277	137-138	—	—	—	—	15.2	15.0
W 112	$C_{19}H_{22}O_2N_4S$	370	89	—	—	—	—	15.1	14.9
W 114	$C_{20}H_{11}O_2N_4S$	391	142 (decomp.)					16.4 <sup>a</sup>	16.1 <sup>a</sup>
W 115	$C_{18}H_{15}O_2N_4S_2$	397	168 (decomp.)					17.6	17.0
W 117	$C_{18}H_{15}O_2N_4S_2$	412	196 (decomp.)					20.4	20.1
W 134	$C_{24}H_{22}O_2N_4S$	446	181 (decomp.)					12.5	12.3
W 138	$C_{19}H_{21}O_2N_4SCl$	404.5	203 (decomp.)	56.4	57.0	5.2	5.5	—	—
W 139	$C_{20}H_{24}O_2N_4S$	384	106	62.5	62.0	6.3	6.8		
W 113	$C_{15}H_{13}ON_3$	263	180					15.9	15.6

<sup>a</sup> For the chlorhydrate  $C_{20}H_{15}O_2N_4SCl$

bacteriostatic effects in any of the substances concerned. However, they revealed interesting facts concerning the influence exerted by the substituents. The compound W 134 proved to be the most active substance in the series. Structural changes in the indole ring, such as the elimination of the methyl group and the introduction of a second methyl group or a chlorine atom, would seem to reduce the activity.

More detailed results of the bacteriological investigations will be published later (5, 6).

#### ACKNOWLEDGMENT

We take the opportunity of conveying our thanks to "Svenska Nationalföreningen mot Tuberkulos" which supported our researches by grants.

#### SUMMARY

Several 2-methylindole-3-azo compounds were prepared to test their bacteriostatic activity against *Mycobacterium tuberculosis*. Certain preliminary results have been discussed.

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# The *in vitro* Reversibility of Cholinesterase Inhibition by Diisopropyl Fluorophosphate (DFP)<sup>1,2</sup>

David Nachmansohn, Mortimer A. Rothenberg  
and Emily A. Feld

*From the Departments of Neurology and Biochemistry, College of  
Physicians and Surgeons, Columbia University, New York*

Received April 25, 1947

## INTRODUCTION

It is generally agreed that the nervous impulse is propagated by small electric currents. The energy for these currents cannot be derived from the stimulus itself, as *e.g.*, in the case of a sound wave, but has to be supplied locally (the "progressive disturbance" of Keith Lucas (1)). The most probable assumption of the mechanism underlying such a disturbance is that of a series of chemical reactions leading to a rapidly reversible change of the active membrane. A great variety of observations have been described which indicate that the release and removal of acetylcholine are events in the surface membrane necessary for the conduction in nerve and muscle (2-4). These investigations were based on the study of the enzyme mechanisms connected with the formation and hydrolysis of acetylcholine and the association of the enzyme activity with physically recorded events in the intact cell. One of the most outstanding features found in the enzyme studies is the high speed with which the active ester can be split by cholinesterase. The prerequisite for any assumption correlating a chemical reaction with conduction is a rate of this reaction which parallels that of electrical manifestations. Other important features of the enzyme are its exclusive localization in the neuronal surface where the bioelectrical phenomena occur, its ubiquity in all conducting mechanisms through-

<sup>1</sup> Dedicated to Professor Carl Neuberger on his 70th birthday.

<sup>2</sup> The work described in this paper was carried out under a contract between The Medical Division, Chemical Corps, U. S. Army, and Columbia University, and was supported by a grant from the Josiah Macy, Jr., Foundation.



out the whole animal kingdom (4), and its relative specificity for acetylcholine in contrast to other esterases occurring in the body (5).

The activity of cholinesterase could be associated with the electrical manifestations during nerve activity in several ways. Studies on electric tissue revealed a direct proportionality between voltage and cholinesterase activity suggesting an interdependence between chemical and electrical events (6). It has also been shown that the primary energy released during recovery which accounts for the electric energy released during the discharge, namely, that of energy-rich phosphate bonds, is used for the resynthesis of acetylcholine (7, 8). An enzyme, choline acetylase, could be demonstrated in nerve and muscle tissue which forms acetylcholine in presence of adenosine triphosphate (9-12). These observations suggest that the breakdown of the ester precedes the breakdown of adenosine triphosphate.

If the alterations of the surface membrane during the passage of the impulse require a rapid removal of acetylcholine, then inhibition of the enzyme should interfere and, in sufficiently high concentration, abolish conduction. It has been shown on different types of nerves, warm and cold-blooded, vertebrate and invertebrate, and on striated muscle, that eserine, a powerful cholinesterase inhibitor, abolishes conduction (13-14). This effect is easily reversible as could be expected from the reversibility of the enzyme-inhibitor complex.

Recently a new powerful inhibitor of cholinesterase became known, the diisopropyl fluorophosphate (DFP), which, in contrast to other known cholinesterase inhibitors, destroys cholinesterase irreversibly. The first investigators studying this enzyme inhibition were unable to demonstrate any reversibility *in vitro* (15, 16). Since the abolition of the action potential in nerves exposed to DFP is reversible, at least for a certain length of time, an immediate irreversible destruction of the enzyme appeared incompatible with the assumption of an essential role of the enzyme in conduction. The question whether or not reversibility of the enzyme-inhibitor complex is possible thus became crucial for the interpretation of the effect of DFP on the nerve action potential and beyond that, for the fundamental problem of the necessity of cholinesterase activity in conduction generally.

It has been shown that the inhibition of cholinesterase by DFP can be reversed *in vitro* (17). In this paper, experiments will be described in which the degree of reversibility has been examined as function of temperature and inhibitor concentration.

## METHODS

The enzyme activity was determined by the manometric method as previously described (5). Two enzyme preparations were used, one prepared from electric tissue of *Electrophorus electricus* and one obtained from the nucleus caudatus

of ox. The highly active enzyme solution which may be obtained from the electric tissue was prepared in a manner described recently (18). One cc. of the solution was able to split about 500 mg. of acetylcholine/hr. One mg. of protein in this solution could hydrolyze 300 mg. of the ester/hr. The nucleus caudatus of ox is extremely rich in cholinesterase (19-21). One g. of this tissue splits about 200-300 mg. of acetylcholine/hr. It therefore forms an excellent source for obtaining mammalian cholinesterase specific for acetylcholine (5). The tissue was ground mechanically with silicate according to the method described by Potter and Elvehjem (22). The same suspension was used throughout the whole series of experiments.

## RESULTS

Even if a compound inhibits an enzyme irreversibly, it is possible to assume that the rate of destruction depends on a series of factors, like temperature, concentration of the inhibitor, and affinity to the particular enzyme. Some of these factors will be interdependent.

A convenient method for studying this problem is that of testing the effect of dilution on the stability of the enzyme-inhibitor complex. For a given temperature, an appropriate range of inhibitor concentration has to be found, within which the inhibitory effect on the enzyme at a relatively high concentration may be compared with that at a low concentration. If the inhibition is reversible, the same enzyme activity will be found regardless of whether the concentration is low from the beginning or whether a low concentration is obtained by dilution of an initially high concentration. If, however, the process is irreversible, the dilution will not reactivate the enzyme. In this way it may be found whether or not the destruction is an immediate process or whether it progresses slowly. By exposing the enzyme for varying periods of time to the higher concentration and diluting it, a fair picture of the rate of irreversible destruction may be obtained.

### 1. Cholinesterase from Electric Tissue

The lower the temperature, the lower should be the rate of irreversible inactivation. Since the DFP is an extremely active compound, it appeared advisable to start with a low temperature; 7-8°C. was selected for the first tests. For these experiments, the solution prepared from the electric tissue of *Electrophorus electricus* was used.

All experiments with this solution were carried out in the following way. The enzyme solutions were kept in a thermostable bath during the entire course of the experiments. One part of the enzyme was exposed to a low concentration of DFP, another part to a high concentration. From the latter solution, aliquot parts were taken and diluted,

either immediately or after a certain period of exposure. The time required for taking the solution out of the thermostat, diluting it, putting into the Warburg vessel, gassing and putting back into the thermostat, was kept at the minimum necessary and did not exceed 6-8 min. The first reading was generally made within 15-20 min.

Fig. 1 shows an experiment in which the enzyme was exposed to 4  $\gamma$  of DFP/cc. at 7°C. To 1 cc. of the original enzyme solution diluted

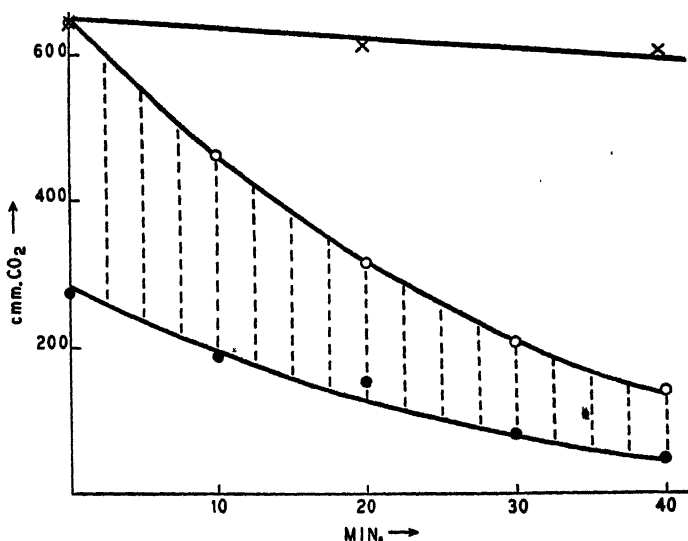


FIG. 1. Reversibility of Cholinesterase Inhibition by DFP at 7°C. Electric tissue esterase. After exposure of the enzyme for varying periods of time to 4  $\gamma$  of DFP/cc., the inhibitor was diluted to 0.13  $\gamma$ /cc.

x-x-x: Diluted from the beginning

●-●-●: Undiluted

o-o-o: Diluted after exposure

three-fold with buffer, was added 1 cc. of a buffer solution containing 8  $\gamma$  of DFP. To this solution was added, either immediately or after varying periods of time, 29 cc. of buffer, either without DFP or containing 4  $\gamma$  of DFP/cc. Three cc. were used for the manometric determination. The activity of this solution was compared with the enzyme solution containing 0.13  $\gamma$ /cc. from the beginning, i.e., 1/30th of the higher inhibitor concentration. The line connecting the crosses shows the slow decrease of the enzyme activity in the solution exposed only to the low DFP concentration. The line connecting the dark circles

shows the activity in the solution containing the undiluted high DFP concentration. The open circles indicate the reactivation obtained by dilution with buffer alone after exposure to the high concentration.

Four  $\gamma$  of DFP is a relatively high concentration, but even so, some reversibility may be obtained even after 40 min. as may be seen in Fig. 1. In the beginning, nearly the total activity can be regained. The shaded area between lower and middle line indicates the reversible

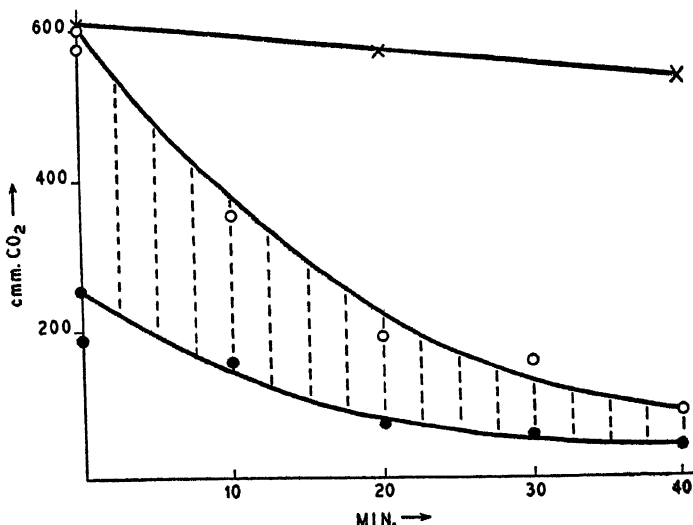


FIG. 2. Reversibility of Cholinesterase Inhibition by DFP at 22.5°C. Electric tissue esterase. After exposure of the enzyme for varying periods of time to 1.2  $\gamma$ /cc., the inhibitor was diluted to 0.04  $\gamma$ . Symbols as in Fig. 1.

part of the enzyme inhibition under these particular conditions. The blank area between the middle and upper line gives a measure of the irreversible inactivation of the enzyme.

In order to obtain a similar curve of reversibility at room temperature (22.5°C.), the enzyme had to be exposed to less than  $\frac{1}{3}$  of the concentration used at 7°C., if all other conditions were kept identical. Fig. 2 shows the data obtained with the same enzyme solution when exposed to 1.2  $\gamma$  of DFP and diluted by the same procedure to 0.04  $\gamma$ .

It may be expected that using the high concentration of 4  $\gamma$ /cc. at 22°C., the conditions for determining reversibility would be unfavor-

able, since the rate of destruction would be too high. On the other hand, if the enzyme were exposed to 1.2  $\gamma$  of DFP/cc. at 7–8°C., reactivation should be possible for a period of time about 3 times as long as that used in Fig. 1. In other words, it may be expected that the two factors, temperature and concentration are interdependent and that the degree of reversibility will vary as a function of both. An experiment is reproduced in Fig. 3 in which the reversibility was tested as a function of

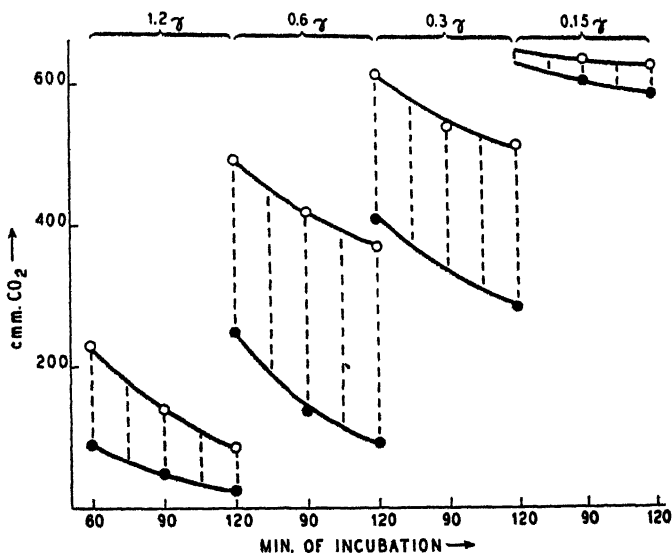


FIG. 3. Reversibility of Cholinesterase Inhibition on Exposure to Varying Concentrations of DFP for 60–120 Min. at 7°C. Electric tissue esterase. Symbols as in Fig. 1.

concentration after longer periods of exposure (from 60 to 120 min.) With 1.2  $\gamma$  of DFP at this low temperature, some reactivation could still be obtained even after 120 min. of exposure comparable to that observed after 40 min. at 23°C., which corresponds to a  $Q_{10}$  of about 2. The maximum degree of reversibility for this period of exposure and at this temperature is, however, obtained with 0.6  $\gamma$  of DFP/cc. The lower the concentration used, the longer will be the period of exposure required for obtaining maximum reversibility. With short exposure times, the maximum will shift to the higher concentration.

## 2. Brain Cholinesterase

For both theoretical and practical reasons, it appeared of interest to test whether cholinesterase could be reactivated *in vitro* after exposure to DFP even at 37°C. Since the esterase obtained from electric tissue is not suitable for experiments at 37°C., being extremely unstable at this temperature, the cholinesterase prepared from the nucleus caudatus of ox has been used.

The procedure was, in principle, the same as described above, with only a few minor differences. To 3 cc. of the enzyme preparation, 1 cc. of buffer solution containing

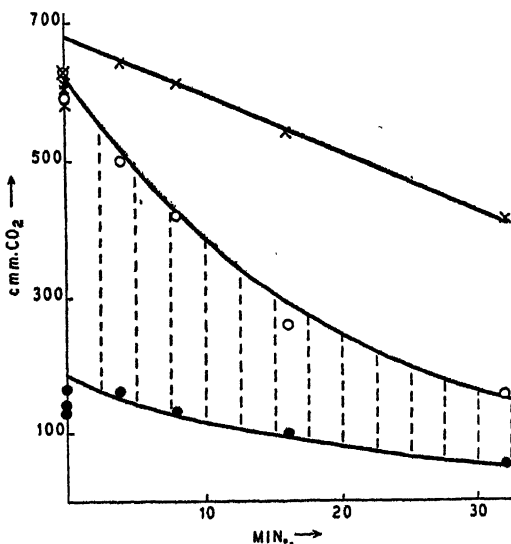


FIG. 4. Reversibility of Cholinesterase Inhibition by DFP at 37°C. Esterase from nucleus caudatus of ox brain. After exposure of the enzyme for varying periods of time to 0.8  $\gamma$  of DFP/cc., the inhibitor was diluted to 0.1  $\gamma$ /cc. Symbols as in Fig. 1.

3.2  $\gamma$  of DFP was added. The final concentration of DFP to which the enzyme was exposed, was thus 0.8  $\gamma$ /cc. After varying periods of exposure, 1 cc. was taken out and 7 cc. of buffer was added, either without DFP or containing 0.8  $\gamma$  of DFP/cc. One cc. of the diluted enzyme preparation was put into the vessel and 3 cc. of buffer was added. The final concentration during the determination in the vessel was consequently only  $\frac{1}{4}$  of that to which the enzyme was exposed, *viz.*, 0.2  $\gamma$  of DFP in the undiluted, 0.025  $\gamma$  of DFP in the diluted preparation.

Fig. 4 shows an experiment in which the brain esterase was exposed to 0.8  $\gamma$  of DFP/cc. at 37°C. The periods of exposure were shorter than

in experiments at lower temperature. Dilutions were made at 4, 8, 16 and 32 min. Even at this high temperature, good reversibility could be demonstrated in the beginning, but after 32 min. irreversible destruction, although not yet complete, had progressed rather far.

In another experiment the brain esterase preparation was exposed to 0.8  $\gamma$  of DFP/cc. at room temperature (23°C.). Fig. 5 shows the data obtained. At this temperature it takes about 80 min. until the effect of reactivation by dilution becomes small. If, however, at the same tempera-

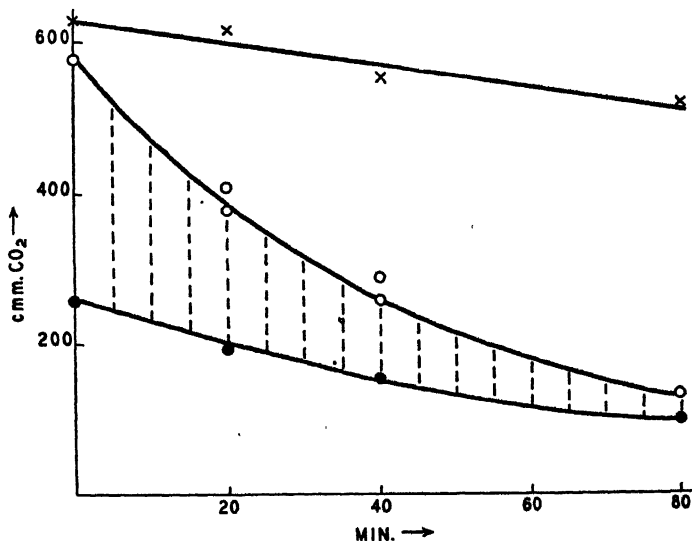


FIG. 5. Reversibility of Cholinesterase Inhibition by DFP at 23°C. Esterase from nucleus caudatus of ox brain. After exposure of the enzyme for varying periods of time to 0.8  $\gamma$  of DFP/cc., the inhibitor was diluted to 0.1  $\gamma$ . Symbols as in Fig. 1.

ture, the concentration of DFP is increased to 2.0  $\gamma$ /cc., the rate of inactivation is more than doubled (Fig. 6). Here, again, as in the case of cholinesterase from electric tissue, the close relationship between the two different factors, temperature and DFP concentration, influencing the rate of destruction, is obvious. In spite of the many and rapid manipulations necessary, the individual values are fairly well reproducible in the initial stages as long as the activity is high. After a certain time of exposure when the enzyme activity has fallen to low values, the variations between the individual determinations become relatively

greater. At that range however, the reversibility is small and, therefore, the individual differences less significant. An example is given in Table I which shows the data of an experiment in which the brain cholinesterase was exposed to 1  $\gamma$  of DFP/cc. at 23°C.

The data presented permit one to estimate that the  $Q_{10}$  for the irreversible cholinesterase inhibition by DFP is close to 2. This is of importance in connection with the experiments on the parallelism between the reversibility of the action potential in nerves exposed to DFP

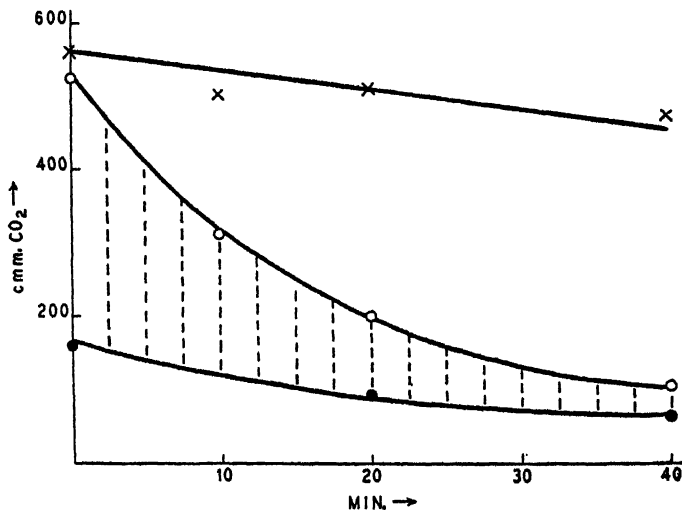


FIG. 6. Reversibility of Cholinesterase Inhibition by DFP at 23°C. Esterase from nucleus caudatus of ox brain. After exposure of the enzyme for varying periods of time to 2.0  $\gamma$  of DFP/cc., the inhibitor was diluted to 0.1  $\gamma$ . Symbols as in Fig. 1.

and the reversibility of the cholinesterase activity. Such a parallelism was first established as a function of time (17). In Table II are summarized some experiments carried out at different temperatures with various nerve preparations exposed to similar concentrations of DFP. The period of time required for irreversible abolition of conduction is consistent with that which might be expected from the irreversible destruction of cholinesterase on the basis of a  $Q_{10}$  of 2. At 37°C. conduction is abolished irreversibly in 20–30 min. At room temperature (18–23°C.) the time required is about 100–120 min., and it is more than



200 min. at 7–8°C. Since different nerve preparations were used, there are obviously other factors involved, the most important probably being the character and thickness of the lipid membrane surrounding

TABLE I

*In vitro Reversibility of Cholinesterase Inhibition by DFP*

Brain esterase prepared from nucleus caudatus of ox is exposed to 1.2  $\gamma$  of DFP/cc. for varying periods of time. After exposure to this concentration, solution is diluted 8 times and compared with the enzyme activity exposed to only 0.15  $\gamma$  of DFP/cc. from the beginning;  $t = 23^\circ\text{C}$ .

Time of exp.  <i>min.</i>	Mm. <sup>3</sup> CO <sub>2</sub> output/hr.				Exposed to 0.15 $\gamma$
	Exposed to 1.2 $\gamma$		Diluted from 1.2 to 0.15 $\gamma$		
	<i>s</i>	<i>a</i>	<i>s</i>	<i>a</i>	
0	272 258	265	560 558	559	615
10	216 207	210	388 386	387	585
20	161 180 178	173	271 262 340	291	547
40	78 114 65 72 136	93	146 152 150 195 204	169	566
80	70 76 40 65	63	84 80 130	98	446 419

the axon since DFP is known to be more soluble in lipid than in water. All the more, the agreement obtained between the  $Q_{10}$  of irreversible enzyme destruction and the effect of temperature on irreversible abolition of conduction appears to be satisfactory.

TABLE II

*Reversibility (=R) of the Action Potential in Nerves Exposed to DFP  
for Varying Periods of Time at Different Temperatures*

T = time of exposure in min.

37°C.		20-23°C.				7-8°C.	
Sup. cervical symp. cat		Sciatic bullfrog		Abdominal chain lobster		Abdominal chain lobster	
T	R	T	R	T	R	T	R
2	++++	25	++++	30	++++	50	++++
7	+++	43	++++	60	+++	100	++++
23	—	85	+++	90	+	150	++++
		120	—	120	—	200	++

## DISCUSSION

The essential facts which emerge from the data presented are the possibility of the reversal of cholinesterase inhibition *in vitro* and the dependence of the rate of irreversible destruction on temperature, on concentration of the inhibitor and length of exposure. The inhibitor concentration at which reversibility of the enzyme inhibition may be demonstrated is of the order of magnitude of a few micrograms/cc. But even at this low concentration and low temperature of 7°C., the maximum reversibility has a rather narrow range, as can be seen from Fig. 3. If, *e.g.*, the reversibility would be tested only after 2 hours, it would not be detected with concentrations of DFP above 1  $\gamma$  or below 0.2  $\gamma$ /cc. Thus, even slight changes of the optimal concentration, which seems to be close to 0.3–0.6  $\gamma$ ., lead rapidly to a fall in reversibility. The rate of destruction is greatly increased at higher temperatures. The factor is 3 for 15°C. which corresponds to a  $Q_{10}$  of 2. The rate may be lowered, however, by using correspondingly lower concentrations of the inhibitor.

These observations are consistent with the high toxicity of the compound and explain the difficulty of earlier investigators to demonstrate the reversibility after injection of the compound into warm-blooded animals. Their failure in this respect was considered as a serious difficulty for the assumption of an essential role of cholinesterase activity in conduction since the abolition of conduction may be reversed for a certain length of time. This objection has now been eliminated.

Undoubtedly there are other factors which may be of importance for the reversibility of the enzyme inhibition by DFP. This compound is an inhibitor of all esterases and not only of cholinesterase. The affinity of the different esterases, whether specific cholinesterase or one of the unspecified esterases, seems however, to vary considerably and may, therefore, influence the rate of irreversible inactivation. Another factor which presumably will be of importance is the state of the enzyme when exposed to the inhibitor, *viz.*, whether the enzyme is in purified solution, or in the presence of a large amount of other proteins and enzymes possibly reacting with the inhibitor, or in a homogenized suspension, or finally in an intact cell or tissue surrounded by membranes of varying permeability for the compound.

This leads us to the interesting and physiologically important question, *viz.*, that of the concentration necessary to destroy the enzyme *in vitro* and to abolish conduction in the intact nerve. *In vitro* complete enzyme inhibition may be obtained with a concentration of the order of magnitude of micrograms, in the experiments on the intact nerve, of the order of milligrams. The outside concentration gives no indication of the concentration of inhibitor present at the site and time of action. The lipid membrane surrounding all axons, "unmyelinated" as well as "myelinated," may form a barrier beyond which the concentration may differ to a very high degree from that of the external solution. For example, when the giant axon of squid was exposed to 1 mg. of DFP/cc., only 0.6% of the outside concentration was found to be present in the axoplasm extruded at the time when the action potential was completely abolished. This corresponds to 6  $\gamma$ /cc. The exposure time required was 12 min. at room temperature. Although it may be objected that, in the protoplasmic membrane surrounding the axoplasm, the concentration of DFP may be different from that found in the axoplasm, it is difficult to conceive that, in the phase beyond the lipid membrane, the differences are considerable. The order of magnitude of the inhibitor concentration necessary for complete or nearly complete inhibition of the enzyme within the period of time observed, appears thus to be in satisfactory agreement with that found in the *in vitro* experiments.

Another important observation in this connection is that of Roeder *et al.*, on the difference of concentration of DFP required to abolish axonal conduction and synaptic transmission (23). Whereas synaptic transmission was blocked by a concentration of  $6 \times 10^{-4}$  M of DFP,

axonal conduction was not affected by a 10-times higher concentration. It has been pointed out repeatedly that synaptic junctions may be affected by the lipid-insoluble methylated quarternary ammonium salts, like acetylcholine, prostigmine, and curare, whereas these same compounds do not affect the axon. This suggests, in agreement with neuro-anatomical observations, that synaptic regions may not have a lipid membrane protecting them against these compounds. The peculiar ability of the synapse to react to certain chemical compounds which do not affect axons may thus be attributed to the difference in anatomical structure rather than to a basic difference of the physico-chemical mechanism of propagation. But in the case of compounds which do penetrate the lipid layer and are capable of acting both on axonal conduction and synaptic transmission, quantitative differences may still be expected, especially when the compounds are more soluble in lipids than in water. It is, therefore, not surprising that at the synapse, where DFP may act on the active surface directly without having to cross the lipid layer, a much lower concentration is sufficient to produce block than in the axon. This is in good agreement with the observation on the giant axon of squid mentioned above and the *in vitro* inhibition described here.

#### SUMMARY

The *in vitro* reversibility of cholinesterase inhibition by DFP has been tested. It is shown that the inhibition is reversible for a certain length of time dependent on temperature and on inhibitor concentration.

(1) On exposure of cholinesterase obtained from electric tissue of *Electrophorus electricus* to 4  $\gamma$  of DFP at 7°C., reactivation can be obtained for about 40 min.

(2) At the same temperature, the rate of irreversible destruction of the enzyme is decreased by a factor of 3 if only about  $\frac{1}{3}$  of the concentration of the inhibitor (1.2  $\gamma$ /cc.) is used.

(3) On exposure to 1.2  $\gamma$  of DFP/cc. at a temperature which is 15° higher (22.5°C.), the rate of destruction is about 3 times as high as at 7°C. indicating a  $Q_{10}$  of 2.

(4) For a given temperature, the optimum reversibility depends on the inhibitor concentration used. This optimum seems to have a rather narrow range.

(5) *In vitro* reversibility of DFP inhibition has also been demonstrated at 37°C. with cholinesterase obtained from the nucleus caudatus of ox. Variations of temperature or inhibitor concentration result in changes of reversibility corresponding to those observed with electric tissue esterase at low temperatures.

(6) If the period of time required for irreversible abolition of conduction in various nerve preparations at different temperatures is compared with the rate of irreversible destruction of cholinesterase at the same temperatures, a close parallelism is obtained. In connection with all the other facts known, the observations are further evidence that the abolition of conduction in nerves exposed to DFP must be attributed to the inactivation of cholinesterase.

(7) The *in vitro* inhibition of cholinesterase requires micrograms of DFP; the block of axonal conduction, milligrams. The apparent discrepancy may be attributed to the presence of a lipid membrane surrounding the axon which acts as a barrier and leads to a considerable difference between inside and outside concentration at the time of action.

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The Antistaphylococcal Effect of Penicillin,  
Streptomycin, and 5,7-Dichloro-8-  
hydroxyquinaldine (Sterosan)  
*in Vitro*<sup>1</sup>

Julius Hirsch and Sabire Dosdogru

*Aus dem Hygienischen Institut der Universität, Istanbul, Türkei*

Received May 6, 1947

EINLEITUNG

Baktericidie und Bakteriostase sind die beiden Begriffe, die im allgemeinen zur Kennzeichnung der Wirkung antibakterieller Präparate (Desinfizientien und Chemotherapeutika) dienen. In der grossen Reihe der Publikationen, welche Angaben über den Wirkungsmodus der beiden Antibiotika, Penicillin und Streptomycin, enthalten, wird sowohl von baktericiden als auch von bakteriostatischen Effekten gesprochen. Ähnliche widersprechende Angaben sind in Mitteilungen über die Wirkung anderer Chemotherapeutika (z. B. Sulfonamide) zu finden. Solche Unsicherheiten kommen zustande, wenn man z.B. mit dem Begriff der Baktericidie nicht nur die direkte Abtötung der Keime bezeichnet, sondern auch das spontane Absterben nicht vermehrungsfähiger Kulturen, indem man diesen sekundären Effekt als wesentliche Endwirkung bewertet. Ebenso unsicher wird der Begriff der Bakteriostase, wenn man z.B. die zeitlich verzögerte Entwicklung einer Kultur, deren Population durch ein Zellgift nicht quantitativ abgetötet ist, als einen Hemmungseffekt bezeichnet. Solche Verwirrungen werden vorwiegend durch einmalige Stichproben (Aussaaten in Subkulturen) verursacht, die keineswegs ausreichen, um einen antibakteriellen Wirkungsmodus zu analysieren. Die exakte Kennzeichnung eines antibakteriellen Effektes kann nur mit Prüfungsmethoden erzielt werden, die es gestatten, die Wirkung unmittelbar nach Zusatz des Präparates abzulesen und die weiteren Reaktionen der Mikroorganismen fortlaufend zu verfolgen.

<sup>1</sup> Carl Neuberg zum 70. Geburtstag.



In den folgenden Experimenten haben wir den Effekt von Penicillin, Streptomycin und 5,7-Dichlor-8-oxychinaldin (Sterosan) auf Staphylokokken-Suspensionen und Kulturen—unter identischen Bedingungen—einer Prüfung unterzogen. Diese vergleichende Analyse soll einen Beitrag zur Kenntnis der Wirkungstypen antibakterieller Effekte liefern.

### EXPERIMENTELLES

*Methoden und Material.* Vor 10 Jahren hat der eine von uns (H) gezeigt, dass die manometrische Messung der Atmungsgrösse von Bakterienkulturen in der Barcroft-Warburg-Apparatur eine brauchbare Methode liefert, um die Vermehrung aerober Mikroorganismen zu analysieren (1). Dieses gilt besonders für die logarithmische Phase der Vermehrung. In dieser Hauptphase erfolgen die Steigerung der Keimzahl und die Steigerung der Atmungsgrösse (= Sauerstoffverbrauch pro Zeiteinheit) mit der gleichen Geschwindigkeit.

Die fortlaufende Bestimmung der Atmungsgrösse proliferierender Kulturen kann auch zur qualitativen und quantitativen Ermittlung der Wirkung antibakterieller Substanzen dienen (2–6). Der Effekt eines zugesetzten Präparates wird durch die Abweichung der Atmungskurve von der Kurve der Kontroll-Kultur gekennzeichnet. Hierbei ist mit der Möglichkeit zu rechnen, dass Störungen der Atmung aerober Kulturen auf einer selektiven Schädigung von Atmungsfermenten beruhen, ohne dass andere physiologische Leistungen der Zellen direkt beeinträchtigt werden. Solche selektive Störungen des Atmungsprozesses lassen sich ohne weiteres durch Prüfung des Präparates an dem System "ruhender" (resting), nicht proliferierender Keime nachweisen. Wird die Atmung "ruhender" Mikroorganismen durch Zusatz eines Stoffes nicht verändert, so ist die Möglichkeit einer spezifischen Schädigung der Atmungsfermente auszuschliessen. Darüber hinaus beweist ein refraktäres Verhalten "ruhender" Bakterien im Respirationsversuch, dass die geprüfte Substanz keinen unmittelbaren baktericiden Effekt ausübt; anderenfalls müsste sie die Atmung "ruhender" Zellen absenken bzw. sistieren. Die manometrische Analyse eignet sich besonders für die Prüfung von Präparaten, welche—wie die meisten modernen Chemotherapeutika—nur die Keimvermehrung stören, ohne die Mikroorganismen durch desinfektorische Vergiftung direkt abzutöten.

Als Testkeim für die folgenden Experimente diente ein Laboratoriums-Stamm von *Staph. pyogenes aureus*. Die Nährlösung der Testkulturen hatte folgende Zusammensetzung: 1% Difco Bacto-Peptone, 0.2% Marmite, 0.3% Glucose, 0.5%  $\text{Na}_2\text{SO}_4 \cdot 10 \text{ H}_2\text{O}$ , 0.01%  $\text{MgCl}_2$ , 0.066 M Phosphatpuffer pH 7.0. Das Impfmateriel lieferten 14-stündige Kulturen in der gleichen Nährlösung; Einsaat 1:70. Suspensionen "ruhender" Staphylokokken wurden folgendermassen hergestellt: 14-stündige Kulturen wurden zentrifugiert. Die Keime wurden in dem doppelten Volumen einer Lösung suspendiert, welche 0.5%  $\text{Na}_2\text{SO}_4 \cdot 10 \text{ H}_2\text{O}$ , 0.01%  $\text{MgCl}_2$  und 0.066 M Phosphatpuffer (pH 7.0) enthielt. Nach nochmaligem Auswaschen mit der gleichen Lösung wurde eine solche Verdünnung der Kokkensuspension hergestellt, dass nach Zusatz von 0.1 M Natriumlactat eine Atmungsgrösse von etwa 20–30 mm.<sup>3</sup>  $\text{O}_2$  pro ml. in 30 Minuten erzielt wurde.

*Geprüfte Präparate.* Penicillin—C.S.C., Crystalline Sodium Salt, Potency 1515 Units/mg., Lot No. 46042402. Streptomycin Merck (Hydrochloride) Lot No. 556 (1.66 g. equivalent 1 g. Streptomycin Base). 5,7-Dichlor-8-oxychinaldin (Sterosan, J. R. Geigy A.-G. Bâle); das Präparat ist in Wasser sehr wenig, dagegen etwas mehr in verdünnten Säuren löslich. Die gelösten Präparate wurden in die seitlichen Retorten von Warburg-Gefäßen eingefüllt und zur gegebenen Zeit den Kokken-Suspensionen bzw. Kulturen zugesetzt. Die Ergebnisse der manometrischen Messungen wurden im halblogarithmischen Koordinatensystem aufgezeichnet.

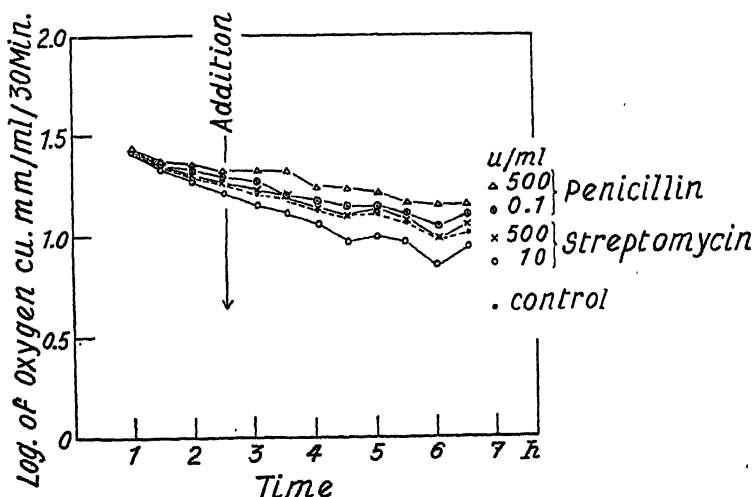


FIG. 1. Die Wirkung von Penicillin und Streptomycin auf die Atmung "ruhender" Staphylokokken (Atmungs-Substrat: 0.1 M Natriumlaktat).

### ERGEBNISSE

*Die Wirkung von Penicillin und Streptomycin auf die Atmung "ruhender" Staphylokokken (Fig. 1).* Die Atmung "ruhender" Staphylokokken in Milchsäure als Atmungssubstrat zeigt im Laufe von 6–7 Stunden einen langsamen Abfall auf etwa die Hälfte des Anfangswertes der Atmungsgrößen. Der Zusatz von Penicillin (0.1 und 500 U./ml.) und von Streptomycin (10 und 500 U./ml.) ist ohne Einfluss auf die Atmung "ruhender" Kokken. Die Atmungskurven von Suspensionen, die den Zusatz der Antibiotika 2.5 Stunden nach Beginn des Versuches erhalten, weichen nicht von dem Verlauf der Atmungskurve der Kontroll suspension ab. Penicillin und Streptomycin stören nicht die Atmung der Staphylokokken; sie wirken also auch nicht baktericid.

Die Wirkung von Sterosan auf die Atmung "ruhender" *Staphylokokken* (Fig. 2). Mengen von 3.6–22.2  $\gamma$ /ml. Sterosan sind ohne Einfluss auf die Atmung "ruhender" *Staphylokokken*. Nur mit der grössten geprüften Dosis von 55.6  $\gamma$ /ml. wird in der ersten halben Stunde eine besondere Senkung der Atmungsgrösse erzielt. Diese Senkung ist nur vorübergehend, sodass die Atmungskurve in den folgenden 4 Stunden von der Kurve der Kontroll-Suspension nicht abweicht. Eine wesentliche, irreversible Störung der Atmung wird durch Sterosan nicht bewirkt, ebensowenig ist ein baktericider Effekt nachzuweisen.

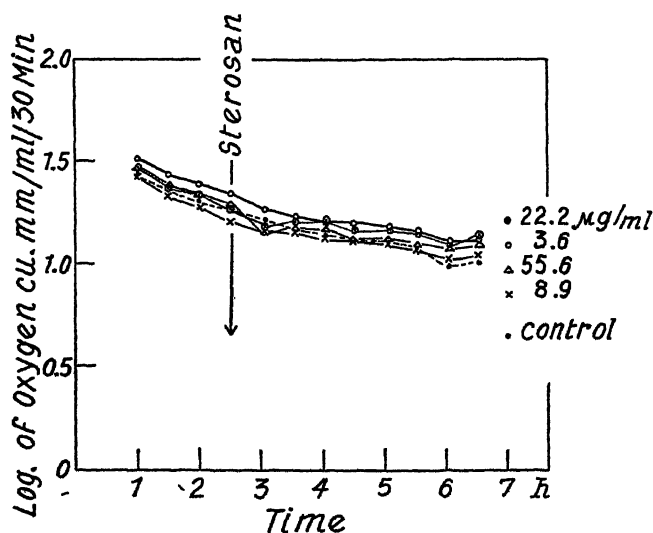


FIG. 2. Die Wirkung von 5,7-Dichlor-8-oxychinaldin (Sterosan) auf die Atmung "ruhender" *Staphylokokken* (Atmungs-Substrat: 0.1 M Natriumlaktat).

Die Wirkung von Penicillin auf die Atmung von *Staphylokokken*-Kulturen während der logarithmischen Phase der Vermehrung (Fig. 3). Unter den gewählten Versuchsbedingungen treten die ersten messbaren Atmungsgrössen 1–1.5 Stunden nach Beginn der Züchtung auf. Gleichzeitig beginnt der logarithmische Anstieg der Atmungskurven, der bei der Kontroll-Kultur in der 5. Stunde beendet ist. Der Zusatz von 0.01 U. und 0.02 U./ml. Penicillin in der Mitte der logarithmischen Vermehrungsphase hat keine Wirkung.

Auf den Zusatz von 0.05 U./ml. folgt zwei Stunden später ein Sistieren der Atmungssteigerung: die Atmungskurve biegt in die Horizontale ein. Anschliessend beginnt sofort ein steiler logarithmischer Abfall der Atmungsgrösse bis in das Bereich nicht messbarer Werte. Der gleiche Effekt wird mit 0.1 U./ml.—jedoch 0.5 Stunde früher—erzielt.

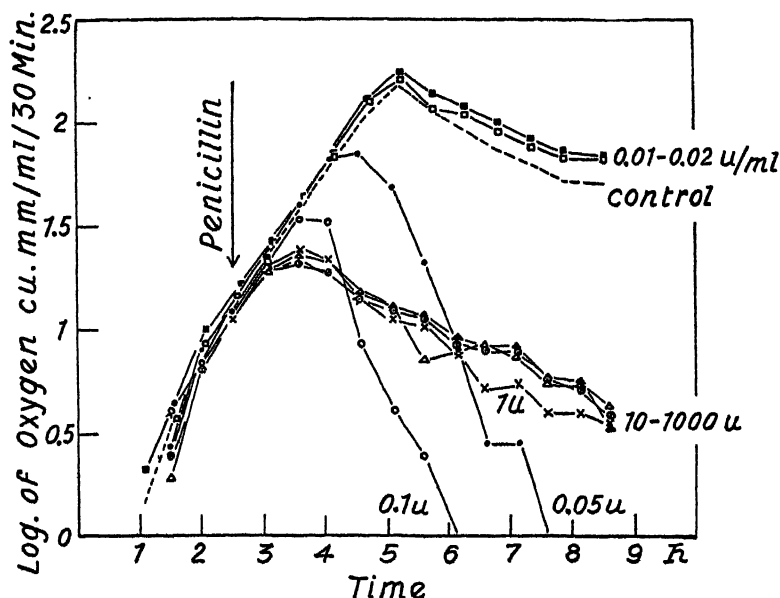


FIG. 3. Die Wirkung von Penicillin (Natriumsalz C.S.C) auf die Atmung von Staphylokokken-Kulturen während der logarithmischen Phase der Vermehrung.

Die Effekte höherer Dosen (1.0 U., 10 U., 100 U. und 1000 U./ml.) zeigen untereinander keinen wesentlichen Unterschied, jedoch ist ihr Wirkungstyp von dem der beiden geringeren Dosen (0.1 U. und 0.05 U.) offensichtlich verschieden. Bereits 1 Stunde nach Zusatz von 1.0 bis 1000 U. hört die Steigerung der Atmung auf, aber die nachfolgende Senkung der Atmungskurven verläuft so langsam, dass das Kurvenbündel von den steiler abfallenden Kurven (0.1 U. und 0.05 U.) geschnitten wird.

Die Geschwindigkeit, mit der die Atmungsgrössen absinken, kann zahlenmässig durch die Zeit ( $x$ ) definiert werden, in welcher der

halbstündige Sauerstoffverbrauch/ml. Kultur auf den halben Wert reduziert wird.

$$x = T \frac{\log 2}{\log O_I - \log O_{II}}$$

$T$  ist die Zeit in Minuten, in der die Atmungsgrösse  $O_I$  auf den Wert  $O_{II}$  absinkt. Die Halbierungszeiten ( $x$ ) der Atmungsgrössen, welche durch die geprüften Penicillin-Einheiten verursacht werden, sind in der Tabelle I zusammengestellt.

TABELLE I  
*Halbierungszeiten der Atmungsgrössen nach Zusatz von  
0.05 bis 1000 U./ml. Penicillin*

Penicillin/ml.	0.05 U.		0.1 U.		1.0 U.		10 U.	100 U.	1000 U.
	Zeit	mm. <sup>3</sup>	Zeit	mm. <sup>3</sup>	Zeit	mm. <sup>3</sup>	mm. <sup>3</sup>	mm. <sup>3</sup>	mm. <sup>3</sup>
$O_I$	4 <sup>h</sup> 37	70.3	4 <sup>h</sup> 05	33.3	4 <sup>h</sup> 05	22.1	21.2	18.7	18.7
$O_{II}$	6 <sup>h</sup> 39	2.8	5 <sup>h</sup> 37	2.5	8 <sup>h</sup> 39	3.6	4.3	3.9	3.4
$T$ (Minuten)	122		92		272				
Halbierungszeit	26 Min.		24 Min.		104 Min.		119 Min.	121 Min.	111 Min.

Aus den Halbierungszeiten der Atmungsgrössen geht hervor, dass mit 0.05 U. und 0.1 U./ml. Penicillin die Atmung der Staphylokokken-Kulturen 4–5 mal schneller absinkt als mit 1.0–1000 U. Zur Deutung dieses paradoxen Phaenomens muss folgender Befund verzeichnet werden: Die Kulturen, welche einen Zusatz von 0.05 und 0.1 U. erhalten haben, zeigen am Ende des Versuches (nach 9 Stunden) eine schwache Opalescens, während die Kulturen mit höheren Penicillin-Dosen eine deutliche Trübung aufweisen, die jedoch nicht an die dichte Trübung der Kontroll-Kultur heranreicht.

*Die Wirkung von Streptomycin auf die Atmung von Staphylokokken-Kulturen während der logarithmischen Phase der Vermehrung (Fig. 4).* Der Zusatz von 2.5 U./ml. Streptomycin während der logarithmischen Vermehrung bewirkt keine Abweichung der Atmungskurve von dem Verlauf der Kontrollkurve. 5 U. bewirken, dass 1.5 Stunden später die Atmungskurve sich verflacht und entsprechend später als die Kontrollkurve ihren Kulminationspunkt erreicht. 10 U. lassen nach einer Latenzzeit von 1 Stunde die Atmungskurve in die Horizontale einbiegen; die Atmungsgrössen bleiben in den folgenden 6 Stunden konstant.

Mit höheren Konzentrationen (20 U., 50 U., 100 U. und 500 U./ml.) wird ein ähnlicher Effekt erzielt, der nach Massgabe der Konzentration früher oder später eintritt. Im ganzen verlaufen die Atmungskurven mit einer geringen Senkung gegen die Abszisse. Die Kurven der Kulturen mit 50–500 U./ml. liegen nahe bei einander; mit 50 U. wird offensichtlich ein maximaler Streptomycin-Effekt erreicht.

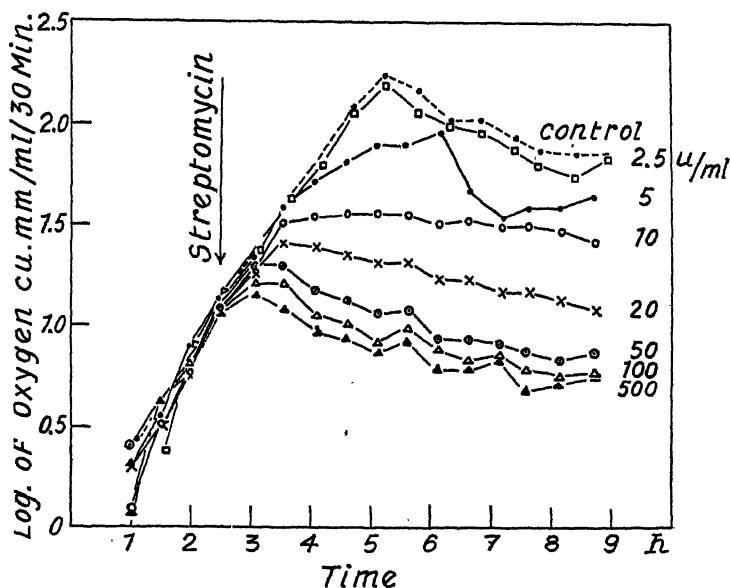


Fig. 4. Die Wirkung von Streptomycin auf die Atmung von Staphylokokken-Kulturen während der logarithmischen Phase der Vermehrung.

Die antibakterielle Aktivität von 5,7-Dichlor-8-oxychinaldin (Sterosan) gegen Staphylokokken und andere grampositive und gramnegative Mikroorganismen. Die Bestimmung der minimalen wirksamen Sterosankonzentrationen gegen verschiedene Bakterien wurde in Reagenzglaskulturen nach dem Verdünnungsverfahren durchgeführt. In diesen ausgedehnten Versuchsreihen wurden die Konzentration des Präparates (1:1) und gleichzeitig auch die Einsaat der Keime (1:10) gestaffelt. Als Nährmedium diente eine nicht gepufferte Lösung von 1% Pepton Witte, 0.2% Marmite, 1.0% Glukose, 0.5%  $\text{Na}_2\text{SO}_4 \cdot 10 \text{ H}_2\text{O}$ , 0.01%  $\text{MgCl}_2$ , die auf pH 7.2 eingestellt wurde. Als Indikator für die Entwicklung der Kulturen wurde Bromthymolblau zugesetzt. Die

Übersichts-Tabelle II gibt die minimalen Sterosan-Konzentrationen an, die bei Einsaaten von wenigstens  $1 \times 10^6$  Keime/ml. keinen Farbumschlag aufkommen liessen. Sterosan ist gegen grampositive Erreger wesentlich wirksamer als gegen gramnegative Bakterien.

TABELLE II

*Minimale wirksame Sterosan-Konzentrationen gegen grampositive und gramnegative Mikroorganismen in Gegenwart von Pepton und Hefe-Extrakt*

Stamm	Einsaat/ml.	minimale wirksame Konzentration		
		$\gamma$ /ml.	Mol.	Verdünnung 1:
<i>Staph. aureus</i> (1)	$1 \times 10^6$	0.57	$2.5 \times 10^{-6}$	1,760,000
<i>Bact. subtilis</i> (2)	$1 \times 10^6$	0.57	$2.5 \times 10^{-6}$	1,760,000
<i>Strep. haemol.</i> (1892)	$1 \times 10^6$	1.14	$5 \times 10^{-6}$	880,000
<i>Strep. haemol.</i> Dick	$1 \times 10^7$	1.14	$5 \times 10^{-6}$	880,000
<i>Bact. paratyphos.</i> C	$1 \times 10^6$	2.28	$1 \times 10^{-5}$	440,000
<i>Bact. coli commune</i> (1)	$1 \times 10^7$	11.4	$5 \times 10^{-5}$	88,000
<i>Bact. coli commune</i> (1)	$1 \times 10^8$	14.2	$6.2 \times 10^{-5}$	70,000
<i>Bact. typhosum</i>	$1 \times 10^7$	22.8	$1 \times 10^{-4}$	44,000*

\* bei pH 7.2 nicht vollständig gelöst.

Die Wirkung von 5,7-Dichlor-8-oxychinaldin (Sterosan) auf die Atmung von Staphylokokken-Kulturen während der logarithmischen Phase der Vermehrung (Fig. 5). Der Zusatz von 0.22  $\gamma$ /ml. Sterosan ist ohne Wirkung auf den weiteren Verlauf der Atmung proliferierender Staphylokokken. Konzentrationen von 0.57  $\gamma$  aufwärts unterbrechen sofort den Anstieg der Atmungskurven; sie bewirken eine starke Depression der Atmungsgrößen (um 55–80%), die über drei Ableisungsperioden (90 Min.) anhält. Dann beginnt wieder ein Anstieg zu dem ursprünglichen Niveau und -nach Massgabe der zugesetzten Menge des Chinaldinderivates- mehr oder weniger darüber hinaus. Die Atmungskurven der Kulturen mit 3.6  $\gamma$ , 8.9  $\gamma$  und 22.2  $\gamma$ /ml. verlaufen bereits mit sehr geringer Steigung, während die Kurven der Kulturen mit 55.6  $\gamma$  und 139  $\gamma$  einen horizontalen Verlauf einschlagen.

## DISKUSSION

Die beiden Antibiotika, Penicillin und Streptomycin, und das Chinaldinderivat Sterosan haben das gemeinsame Charakteristikum, dass sie die Proliferation von Staphylokokken in pepton- und hefeex-

trakt-haltigen Nährmedien mit sehr kleinen Dosen stören, dass sie aber nicht imstande sind, Staphylokokken-Suspensionen in stickstoff-freien Pufferlösungen abzutöten, denn die Atmung "ruhender" Kokken wird auch nach stundenlanger Einwirkung höherer Dosen nicht geschädigt, geschweige denn unterbunden (Fig. 1 und 2). Die geprüften Präparate greifen den oxydativen Stoffwechsel bzw. die Atmungsfermente der Kokken nicht an. Abweichungen der Atmungskurven von Staphylokokken-Kulturen, die nach Zusatz der Präparate

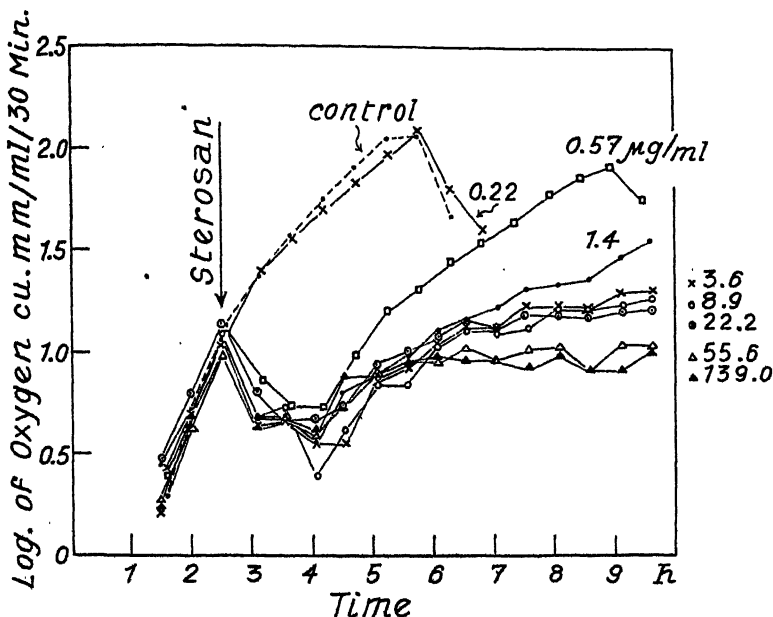


FIG. 5. Die Wirkung von 5,7-Dichlor-8-oxychinaldin (Sterosan) auf die Atmung von Staphylokokken-Kulturen während der logarithmischen Phase der Vermehrung.

in der logarithmischen Phase der Vermehrung auftreten, können nur auf Veränderungen des Proliferationsprozesses beruhen, zumal die Synthese der lebenden Substanz in den belüfteten Kulturen energetisch vorwiegend durch oxydative Prozesse unterhalten wird.

Penicillin, Streptomycin und Sterosan wirken gegen Staphylokokken *nicht baktericid*. Die drei Präparate greifen nur proliferierende Kokken an, aber der Wirkungsmodus gegen Staphylokokken-Kulturen ist verschieden.



Der *Wirkungstyp des Streptomycins* (Fig. 4) ist dem der Sulfonamide (2) ähnlich. Suboptimale Dosen bewirken eine Retardierung der Atmungssteigerung, sie setzen die Vermehrungsgeschwindigkeit herab; optimale Dosen (bei unseren Versuchsbedingungen 10 U./ml. und mehr) unterbrechen die Zunahme der Atmungsgrösse, sie sistieren die Vermehrung der Kokken. Die Atmungskurven verlaufen nunmehr parallel zur Abscisse oder mit einer geringen Senkung, die nicht steiler ist, als die der Atmungskurve "ruhender" Staphylokokken (Fig. 1 und 2). Die Senkung entspricht dem natürlichen Absterbeprozess nicht-proliferierender Zellen. Streptomycin wirkt auf Staphylokokken-Kulturen *bakteriostatisch*. Die Bakteriostase tritt nicht unmittelbar nach Zusatz des Streptomycins ein, sondern nach einer Latenzperiode, deren Dauer von der Grösse der Dosis abhängig ist.

Nach Zusatz abgestufter Mengen von *Penicillin* (C.S.C.) treten *zwei Wirkungstypen* in Erscheinung (Fig. 3). 0.1 U. und 0.05 U./ml. verursachen nach einer Latenzperiode von 1 bzw. 1.5 Stunden, in der die Zunahme der Atmung (Synthese der lebenden Substanz) quantitativ nicht gestört ist, einen steilen logarithmischen Abfall der Atmungskurven, der dem Bild von "Absterbekurven" gleicht, wie sie unmittelbar nach Einwirkung starker Zellgifte auftreten. Wir haben dieses Phänomen in einer früheren Arbeit (5) diskutiert und als "*degenerativen Effekt*" des Penicillins bezeichnet. Es handelt sich um die schnelle Auflösung einer qualitativ geschädigten Kokkenpopulation, die sich in Gegenwart von Penicillin entwickelt hat. Die morphologischen Missbildungen solcher Kokkenkulturen hat Gardner (6) schon 1940 beschrieben.

Mit höheren Dosen des geprüften Präparates (1 U. bis 1000 U./ml.) tritt der degenerative Effekt nicht in Erscheinung. Bereits eine halbe Stunde nach ihrem Zusatz hört die logarithmische Zunahme der Atmungsgrössen auf, und die Kurven sinken in einem geschlossenen Bündel langsam abwärts. Die Wirkung des Präparates mit höheren Dosen gleicht eher dem *bakteriostatischen Effekt* des Streptomycins. Dem entspricht auch die Beobachtung, dass 6 Stunden nach Zusatz von 1 U. bis 1000 U./ml. keine Auflösung der Kokken eingetreten ist, während 0.1 U. und 0.05 U. eine komplette Autolyse der Kulturen auslösen, obwohl deren Proliferation viel später unterbrochen wurde.

Wir haben die beiden Wirkungstypen des degenerativen Effektes kleiner Dosen und des bakteriostatischen Effektes grösserer Dosen früher (5) bei Rohpenicillin und bei einem ungereinigten Bariumsalz

beobachtet, während ein sorgfältig gereinigtes Bariumsalz aus den Wellcome Physiological Research Laboratories (Dr. Trevan) mit allen geprüften Dosen bis zu 50 U./ml. nur den degenerativen Effekt zeigte. Zur Deutung des Phänomens haben wir die Annahme gemacht, dass den ungereinigten Präparaten ein "zweiter antibiotischer Faktor" mit bakteriostatischer Wirkung beigemischt ist. Dabei müsste die Aktivität dieses Faktors auf höhere Konzentrationen der Präparate beschränkt sein und mit dem spezifischen degenerativen Penicillineffekt interferieren. In der Tat gelingt es, den degenerativen Effekt aufzuheben, wenn man gleichzeitig irgend ein bakteriostatisch wirkendes Präparat zusetzt; (z.B. 0.01 M N<sup>1</sup>-3,4-Dimethylbenzoyl-sulfanilamid (Irgafen) (5) oder 10 U./ml. Streptomycin oder 20  $\gamma$ /ml. Sterosan).

Inzwischen sind verschiedene Formen von Penicillin in Kulturen von *Penicillium notatum* identifiziert worden, und es ist bekannt geworden (7) dass die Handelspräparate wechselnde Gemische der 5 bekannten Penicilline F, G, X, K und Dihydro-F enthalten. Die Aktivität (U./mg.) der reinen Präparate ist verschieden: so zeigt kristallisiertes Penicillin X (nach der cup plate-Methode) 900 U./mg. und Penicillin G 1650 U./mg. (8). Über den Wirkungsmodus der verschiedenen Penicilline gegen Staphylokokken und andere Mikroorganismen ist uns bisher nichts bekannt geworden. Immerhin könnte der funktionell nachweisbare "zweite Faktor" mit irgend einem der Penicilline identisch sein, das gegen Staphylokokken nur bakteriostatisch wirkt, während der degenerative Effekt von einem anderen Penicillin ausgehen könnte.

Der Effekt des Chinaldinderivates *Sterosan* auf Staphylokokken-Kulturen (Fig. 5) tritt unmittelbar—ohne Latenzperiode—in Erscheinung; hierin liegt ein wesentlicher Unterschied des Wirkungsmodus des Sterosans von dem der beiden Antibiotika. Schon mit der minimal wirksamen Dosis von 0.57  $\gamma$ /ml. wird der logarithmische Anstieg der Atmungskurve *momentan* unterbrochen. Die plötzliche Sistierung der Vermehrung, das Aussetzen der energetischen Leistung der Plasmasyntese, prägt sich besonders durch den anschliessenden Abfall der Atmungsgrössen aus. Dieser Abfall bedeutet kein Absterben der Kokken, er entspricht der mehr oder minder steilen Senkung der Atmungsgrössen, die auch nach dem natürlichen Abschluss der Hauptphase der Vermehrung in jeder Bakterienkultur festzustellen ist (siehe z.B. Fig. 5; Kontroll-Kultur).

Nach der plötzlichen Stase beginnt ein neuer Anstieg der Atmungsgrößen, der in den Kulturen mit 0.57  $\gamma$  und 1.4  $\gamma$  zu einer mehr oder weniger ausgeprägten Vermehrungskurve führt. Bei diesen geringen Konzentrationen ist in dem vorliegenden Versuch der bakteriostatische Effekt des Sterosans reversibel. Die Atmungsgrößen der Kulturen, denen höhere Konzentrationen (3.6–139  $\gamma$ /ml.) zugesetzt wurden, überschreiten kaum das Niveau des Sauerstoff-Verbrauches, der vor dem Sterosanzusatz erreicht worden war. Ihre Atmungskurven verlaufen über einige Stunden in horizontaler Richtung.

Auffallend ist die Konstanz der Atmung, die nicht einmal bei der Atmung normaler "ruhender Staphylokokken" (Fig. 1 u. 2) festzustellen ist. Wir haben unter gleichen Versuchsbedingungen eine konstante Atmung von Staphylokokken-Kulturen, die durch Sterosan gehemmt waren, über 28 Stunden beobachten können. Die mikroskopische Prüfung zeigt wohlgeformte, aber ausserordentlich grosse Kokkenzellen, sodass mit der Möglichkeit zu rechnen ist, dass Sterosan in erster Linie den Multiplikations-Mechanismus stört und dabei die assimilatorischen Funktionen der einzelnen Zellen nicht vollends unterbindet. Während unter der Einwirkung des Penicillins degenerierte Populationen entstehen, die einer schnellen Autolyse anheimfallen, scheint die *Bakteriostase durch Sterosan* das Leben der einzelnen Zelle sogar zu verlängern.

Sterosan ist—ebenso wie Penicillin und Streptomycin—auch in Gegenwart von Pepton und Hefeextrakt wirksam. Seine antibakterielle Wirkung ist vorwiegend gegen grampositive Erreger gerichtet. Die bakteriostatische Aktivität ( $\gamma$ /ml.) gegen Staphylokokken entspricht der Aktivität des Streptomycins. Die Auswertung nach dem Verdünnungsverfahren zeigt eine minimale wirksame Dosis von 0.57  $\gamma$ /ml. gegen  $1 \times 10^6$  Staphylokokken. Diese Menge gleicht dem Gewicht von 1 U. eines kristallisierten Penicillin-Natriumsalzes mit 1650 U./mg.

#### ZUSAMMENFASSUNG

Der Antistaphylokokken-Effekt von Penicillin, Streptomycin und 5,7-Dichlor-8-oxychinaldin (Sterosan, Geigy) wird durch fortlaufende Messung des  $O_2$ -Verbrauches ruhender Staphylokokken und proliferierender Staphylokokken-Kulturen geprüft. Der Zusatz der

Präparate bewirkt keine Veränderung der Atmung von Staphylokokken in Milchsäure-Phosphatpufferlösung. Penicillin, Streptomycin und Sterosan schädigen nicht die Atmungsfermente der Kokken; sie wirken nicht baktericid.

Sehr kleine Dosen der beiden Antibiotika und des Chinaldinderivates stören die Proliferation von Staphylokokken in pepton- und hefeextrakt-haltigen Nährlösungen; der Wirkungsmodus der Präparate ist verschieden.

Nach Zusatz von Penicillin (Natriumsalz, C.S.C., 1515 U./mg.) während der logarithmischen Phase der Vermehrung treten nach Massgabe der Konzentration zwei Wirkungstypen in Erscheinung. Kleine Dosen (0.05 U. und 0.1 U./ml.) verursachen nach einer längeren Latenzperiode einen steilen Abfall der Atmung, der mit einer Auflösung der Kokken einhergeht. Dieser Typ wird als "degenerativer Effekt" bezeichnet. Grössere Dosen (1 U. bis 1000 U./ml.) bewirken nach einer kürzeren Latenzperiode einen Stillstand der Atmungssteigerung, der einem bakteriostatischen Effekt entspricht; eine Autolyse der Kokken tritt nach grösseren Dosen nicht ein. Die verschiedene und paradoxe Wirkung kleiner und grosser Penicillindosen wird durch das Vorkommen mehrerer Penicilline in den Handelspräparaten zu deuten versucht.

Der Zusatz von 10 U. bis 500 U./ml. Streptomycin bewirkt einen Stillstand der Atmungssteigerung. Die Atmungsgrössen sinken langsam ab, wie die Atmungsgrössen ruhender Staphylokokken. Dieser Effekt tritt nicht unmittelbar nach dem Zusatz in Erscheinung, sondern nach einer Latenzperiode, deren Dauer mit steigender Dosis verkürzt wird. Mit 50 U. bis 500 U./ml. Streptomycin wird der gleiche bakteriostatische Effekt erzielt.

5,7-Dichlor-8-oxychinaldin (0.57  $\gamma$ /ml. und mehr) unterbricht plötzlich—ohne Latenzperiode—den Anstieg der Atmungsgrössen. Der Wirkungstyp des Chinaldinderivates entspricht im übrigen einem bakteriostatischen Effekt. Die Auswertung nach dem Verdünnungsverfahren ergibt eine minimale wirksame Dosis von 0.57  $\gamma$ /ml. gegen  $1 \times 10^6$  Staphylokokken. Sterosan ist gegen grampositive Erreger (Staphylokokken, Streptokokken, Subtilis-Bacillen) wesentlich wirksamer als gegen gramnegative Bakterien (Coli-Typhus-Salmonella Gruppe).

## SUMMARY

The effect of penicillin, streptomycin, and 5,7-dichloro-8-hydroxy-quinaldine (Sterosan) against *Staphylococci* was determined by continuous measurement of the  $O_2$ -consumption of resting microorganisms and proliferating cultures. The addition of the preparations does not cause any change in the respiration of the *Staphylococci* in lactic acid-phosphate buffer. Penicillin, streptomycin, and Sterosan do not harm the respiratory enzymes of the cocci; they are not bactericidal.

Very small quantities of both antibiotics and of the quinaldine derivative upset the proliferation of *Staphylococci* in nutrient solutions containing peptone and yeast extracts. The manner of action of the preparations is different.

Following the addition of penicillin (sodium salt, C.S.C., 1515 U./mg.) two types of action appear during the logarithmic phase of multiplication depending on the concentration. Small quantities (0.05 U. and 0.1 U./ml.), after a considerable latency cause a rapid decrease of respiration, which accompanies the disintegration of the cocci. This type is called the "degenerative effect." Larger quantities (1 U. to 1000 U./ml.) bring about the cessation of the increase of respiration after a shorter latency, which corresponds to the bacteriostatic effect. Autolysis of the cocci does not occur after the addition of larger quantities. It is attempted to interpret the different and paradoxical action of smaller and larger quantities of penicillin on the basis of the presence of several penicillins in commercial preparations.

The addition of 10 U. to 500 U./ml. streptomycin halts the increase of respiration. The respiration quotients decline slowly, like the respiratory quotients of resting *Staphylococci*. This effect does not appear directly after the addition, but after a latency, the length of which is shortened by an increasing quantity. Using 50 U. to 500 U./ml. streptomycin, the same bacteriostatic effect is obtained.

5,7-Dichloro-8-hydroxyquinaldine (0.57  $\gamma$ /ml. and more) suddenly halts the increase of the respiratory quotient without latency. The type of action of the quinaldine derivative corresponds, moreover, to a bacteriostatic effect. Evaluation according to the method of dilution shows the minimum effective quantity against  $1 \times 10^6$  *Staphylococci* to be 0.57  $\gamma$ /ml. This compound is essentially more reactive toward gram positive organisms (*Staphylococci*, *Streptococci*, *Subtilis bacilli*) than toward gram negative bacteria (Coli-Typhoid-Salmonella group).

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On the Mechanism of Enzyme Action. XXIX.  
The Acetate Metabolism of Certain  
Wood-Destroying Molds and the  
Mechanism of Wood Decay

F. F. Nord and James C. Vitucci

*From the Department of Organic Chemistry,<sup>1</sup>  
Fordham University, New York 58, N. Y.*

Received May 5, 1947

INTRODUCTION

The study of the carbohydrate metabolism of the four wood-destroying molds: *Merulius niveus* (Meni), *Merulius tremellosus* (Metre), *Merulius confluens* (Meco), and *Fomes annosus* (Foman) established the formation and accumulation, as metabolic products, of ethyl alcohol, acetaldehyde, acetic acid, and succinic acid (1). Obtainence of acetic acid would be assumed to result either from the dehydrogenation of ethyl alcohol *via* acetaldehyde as in the case of certain acetic acid bacteria (2), since it has been ascertained that an efficient dehydrogenating enzyme system is embodied by these organisms (3), or because of a dismutation of the acetaldehyde. The detection of succinic acid would also point to the presence of such a system, this compound arising through a dehydrogenation of acetic acid.

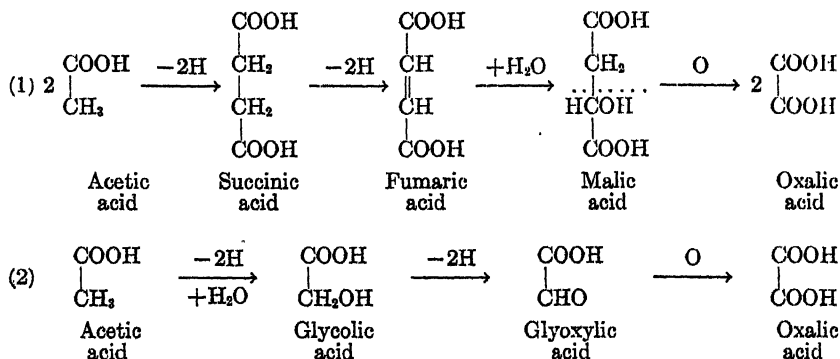
Inasmuch as acetic acid has been previously shown by us to be the last isolable metabolic breakdown product in the carbohydrate metabolism of the above-mentioned organisms, it was thought of interest to investigate the dissimilation of this acid in the form of sodium acetate in order to elaborate the complete phase sequence of wood-cellulose degradation by these molds.

In this attempt the study of the dissimilation of sodium acetate by the above organisms was undertaken and oxalic acid (present as a salt) was found to be the terminal product of the degradation.

<sup>1</sup> Communication No. 54. Dedicated to Professor Carl Neuberg on his 70th birthday, July 29th, 1947, and to thank him for the inspiration received from his work.



Though the fact of the formation of oxalic acid from acetic acid has been established, it remained to clarify the pathway of its origin. The discoloration of resazurin (3) when incorporated in the acetate medium, together with a positive test for succinic acid, while pointing to an oxidation of the latter to give rise to oxalic acid, does not exclude the possible simultaneous formation of the acid through another route, since glycolic acid could also be regarded as a dehydrogenation product of acetic acid. Consequently, it could be assumed that the discoloration of resazurin indicates a dehydrogenation of acetic acid resulting either in succinic acid, glycolic acid, or both. It would be conceivable, then, that two routes can be taken during the formation of oxalic acid, namely:



To elucidate the mechanism of oxalic acid formation three aspects were to be considered: (1) to distinguish whether oxalic acid was formed *via* either or both of these pathways, (2) to establish which intermediary stages were passed during the genesis of the terminal acid, and (3) to attempt to influence the self-regulation in our molds by applying a selectively effective inhibitor.

The solution of the first part was simpler, consisting in the observation of the production or non-production of oxalic acid from succinic and glycolic acids, these acids being forerunners of oxalic acid in the two pathways presented. Two means for the solution of the second part were available: a direct method, consisting of the isolation of the transitory compounds, or an indirect approach, attempting to demonstrate the accumulation or non-obtainment of oxalic acid from possible intermediates on the assumption that failure to produce the acid may

justify the conclusion that the substance does not represent a stage in the phase sequence involved in the pathway to oxalic acid.

The method employed for the solution of both aspects of the problem was to establish the occurrence of oxalic acid and estimate the amount formed when the organisms were allowed to grow on media containing salts of the acids to be investigated as sole carbon sources.

The following groups of acids were employed:

- (a) Two-carbon acids: acetic and glycolic;
- (b) Three-carbon acids: lactic and pyruvic;
- (c) Four-carbon acids: succinic, fumaric, malic, and tartaric;
- (d) Dimethylsuccinic and dimethylfumaric acids.

### EXPERIMENTAL

The wood-destroying molds employed in this investigation were those listed in the introduction and were originally obtained from the New York Botanical Garden through the courtesy of Dr. Wm. J. Robbins.

The media employed were composed of the following:

Organic acid.....	5 or 10 g.
$\text{KH}_2\text{PO}_4$ .....	1.5 g.
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ .....	0.5 g.
Peptone.....	1.5 g.
Thiamine·HCl.....	2 mg.
Tap water to.....	1000 ml.
pH.....	6.3-6.5

The desired amount of acid under study was neutralized with the necessary quantity of alkali. Inoculation was effected by means of spore-mycelial suspensions, and 1 ml. was used in 50 ml. of medium contained in 125 ml. Erlenmeyer flasks; or fully grown mats, prepared as previously described (3), were used. In this case, one mat was transferred to a 250 ml. Erlenmeyer flask containing 100 ml. of the above medium.

The media were sterilized at 20 lbs. pressure for 20 minutes. Sodium pyruvate (4), which could be materially affected by pressure sterilization, was added with sterile technique in a solution of sterile distilled water.

The molds were incubated at 28°C. in the dark. When employing resazurin as an indicator, it was incorporated in the medium prior to sterilization, the concentration used being  $1.5 \times 10^{-5}$  M.

The formation of oxalic acid (obtained as a salt) as a metabolic product of the action of enzymes present in the molds on acetic acid was established as follows: The medium was strongly acidified and steam distilled to remove any volatile acids. The residue, made strongly alkaline with caustic soda, was evaporated *in vacuo*, made strongly acid and exhaustively extracted with ether. After evaporation, a white crystalline product remained. It was recrystallized from water several times to give rise to a well-

defined product having a m.p. of 101°C., and sublimed *in vacuo* giving rise to a compound, m.p. 188–189°C., this being the anhydride of the acid.

A mixed melting point of the recrystallized product and of an authentic sample of oxalic acid (m.p. 100–101°C.) showed no depression, establishing the identity of the product as oxalic acid.

The oxalic acid content was quantitatively estimated as follows: The medium was filtered off, the mycelium washed with hot water, the combined filtrates and washings made distinctly alkaline with ammonia and then acid with acetic acid. The acid was then precipitated as the calcium salt from the boiling solution by addition of a 10%  $\text{CaCl}_2$  solution. After standing for one hour, the insoluble  $\text{CaC}_2\text{O}_4$  was filtered through a Gooch crucible, washed with hot water and the oxalate determined by titration with standard  $\text{KMnO}_4$  in the usual way. The values are calculated as anhydrous  $\text{H}_2\text{C}_2\text{O}_4$  and the theoretical yields based on the assumption that all the carbon of the acid under investigation is converted into oxalic acid.

## RESULTS

### 1. The Mechanism of Oxalic Acid Formation

The previous inability to identify or isolate oxalic acid as a metabolic product when the organisms were allowed to grow on a glucose medium prompted the consideration that perhaps the acid was formed, and, being in a free state, may have been further utilized by the organisms. Thus, *e.g.*, *Aspergillus niger* was cultivated in solutions containing salts of organic acids (5) rather than free acids, since oxalic acid in the absence of another available carbon source was readily dissimilated by the organisms, whereas the acid, present in form of or added as a salt, prevented its further utilization by the fungus.

To establish whether or not these organisms were capable of dissimilating the free acid as well as the fixed acid an experiment was set up with *Meni* cultivated on both substances. The results recorded in Fig. 1 indicate that the fixed acid could not be dissimilated while the free acid was utilized by the fungus. Consequently, it seemed necessary to trap the acid as a salt in order to prove its formation as a metabolic product.

Of the media employed only those containing the acids recorded in scheme I supported growth when inoculated with a spore-mycelial suspension of *Meni*. Spore-mycelial suspensions of the other organism did not allow for growth. Consequently, fully grown mats of the organisms were prepared and used to study their action on the acids. The results of the dissimilation study of the acids employing fully grown mats are presented in Table I.

The findings, as recorded in Table II, also attest to the non-utilization of oxalic acid by these organisms when present as a salt since an accumulation of the acid is indicated. Of the organisms studied, *Meco*, which grew in a submerged state, did not give rise to oxalic acid when grown in media containing the various acids.

Inasmuch as the sodium oxalate was not further utilized by the organisms it was considered unnecessary to make a detailed quantitative study of its production from the acids, but simply to study its

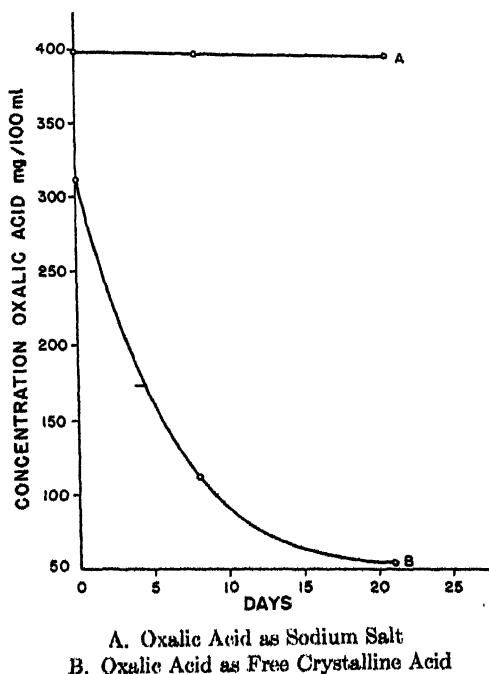


FIG. 1. Dissimilation of Oxalic Acid and Sodium Oxalate by *Merulius niveus*.

quantitative formation from the various acids on any day. The results of such a study are summarized in Table II. The various acids employed are listed and the amounts of oxalic acid formed are shown after the specified incubation period.

All members of the four series of acids mentioned gave rise to substantial quantities of oxalic acid when acted upon by the wood-destroying molds employed. It is to be concluded that the discoloration of resazurin, when incorporated in the acetate, succinate, glycolate,

TABLE I  
*Dissimilation of Acids with Mats*

Acid and organism <sup>1</sup>	Incubation period in days	Oxalic acid <sup>2</sup>
Pyruvic		
Meni	30	7.0
Metre	30	6.8
Meco	30	—
Foman	30	4.6
Lactic		
Meni	40	24.0
Metre	40	16.6
Meco	40	—
Foman	40	7.2
Glycolic		
Meni	25	58.0
Metre	25	19.4
Meco	25	—
Foman	25	8.2
Dimethylsuccinic		
Meni	48	9.55
Metre	48	2.0
Meco	48	—
Foman	48	9.1
Dimethylfumaric		
Meni	14	6.0

<sup>1</sup> Concentration of acids 10 g./l.; pyruvic: 5 g. pyruvate/l.

<sup>2</sup> Oxalic acid expressed in mM.

TABLE II  
*Dissimilation of Acids Mentioned in Schemes with  
Meni Spore-Mycelium Suspension<sup>1</sup>*

Incubation period in days	Acetic		Succinic		Fumaric		Malic	
	M.W. <sup>2</sup>	O.A. <sup>3</sup>	M.W. <sup>2</sup>	O.A. <sup>3</sup>	M.W. <sup>2</sup>	O.A. <sup>3</sup>	M.W. <sup>2</sup>	O.A. <sup>3</sup>
20	92.0	104.0	40.0	18.0	32.0	21.6	33.4	13.6
26	95.8	106.8	—	—	40.0	27.0	34.0	16.8
32	39.4	114.8	40.8	57.2	48.0	63.2	39.4	38.2
40	36.0	132.9	48.6	61.6	66.4	83.4	48.4	47.2
47	27.6	132.9	73.4	64.2	49.2	89.6	36.0	47.0

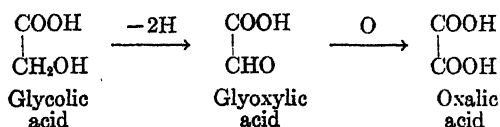
<sup>1</sup> Original concentration of acids 10 g./l.

<sup>2</sup> Mycelial weight expressed in mg./100 ml.

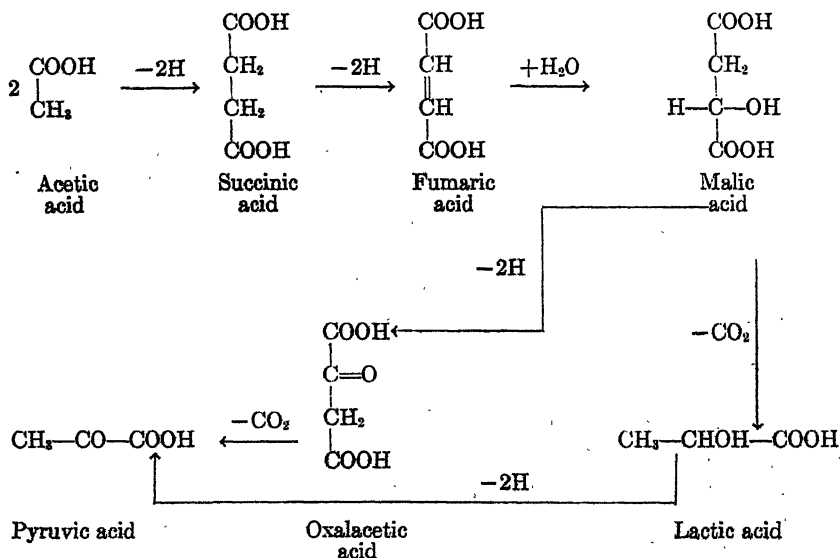
<sup>3</sup> Oxalic acid expressed in mM.

lactate, pyruvate, and dimethylsuccinate media, indicated dehydrogenation of the substrates as a preliminary step in the formation of oxalic acid.

The results attest to the production of oxalic acid from acetic acid *via* both pathways previously indicated. Although glyoxylic acid was not employed as substrate since it can give rise *in vitro*, by application of heat, to oxalic acid (6), the formation of oxalic acid from glycolic acid, together with the discoloration of resazurin when incorporated in the glycolate medium, presumed the intermediate formation of glyoxylic acid as follows:



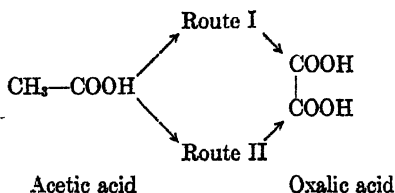
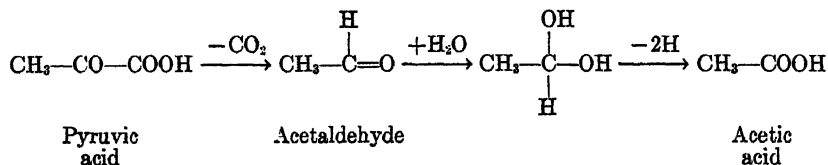
The phase sequence of the genesis of oxalic acid from acetic acid by the action of the wood-destroying molds being established, the arising of oxalic acid from the two members of the three-carbon acid series, lactic and pyruvic acid, prompted the question of the mode of formation of the acid from them. Inasmuch as lactic and pyruvic acids are considered as probably originating from acetic acid in the manner:



it was thought of interest to study the possible role of these acids as substrates.

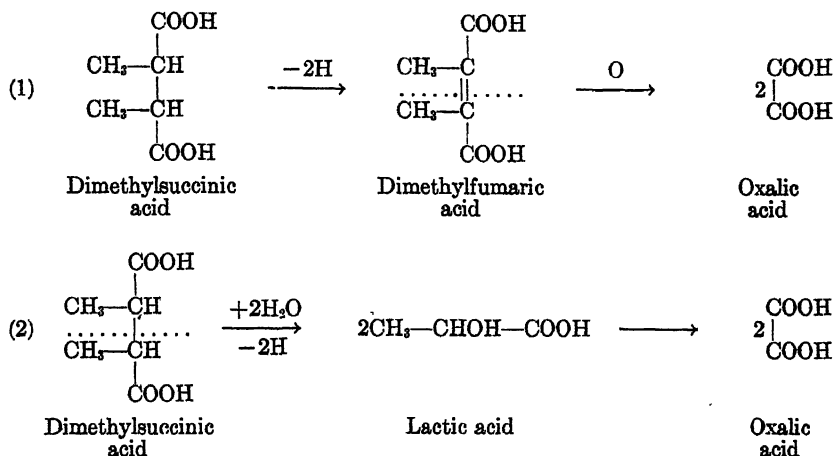
The discoloration of resazurin when incorporated in the lactate and pyruvate media indicates the action of a dehydrogenating enzyme system. The genesis of oxalic acid from pyruvic acid, would suffice also to account for oxalic acid formation from lactic acid, since the discoloration of the dye in the lactate medium points to a dehydrogenation of this acid to give rise to pyruvic acid as an intermediate.

Since thiamine hydrochloride is a necessary medium ingredient, it should be assumed that a decarboxylation of pyruvate takes place with the dehydrogenase system taking part in one or more stages during the course of formation of oxalic acid, probably as follows:



The failure to identify or isolate intermediates of the above scheme does not rule out the possibility of their transitory formation, since it is probable that, under the prevailing conditions, the rates of dissimulation equal or exceed the rates of formation of the intermediates.

The production of oxalic acid from dimethylsuccinic acid by the action of the molds, although of no direct bearing on the acetate → oxalate mechanism, nevertheless prompts an attractive speculation as to the mode of formation of the acid. Bearing in mind that resazurin, when incorporated in the dimethylsuccinate medium, was discolored, a dehydrogenase system must play an important role in such a transformation too. Consequently, two pathways present themselves for consideration:



We were able to demonstrate that dimethylfumaric as well as lactic acid gives rise to oxalic acid.

It is interesting to compare the above findings with those of Raistrick and Clark (5), on the one hand, and those of Walker *et al.* (7) on the other. Our findings attest to the arising of oxalic acid from acetic acid *via two* pathways. In studies with *Aspergillus niger*, the latter investigators demonstrated the passage of the glycolic and glyoxylic acid phase as intermediates in the genesis of oxalic acid from acetic acid while the former authors, working with the same organism failed to do so and thought that acids appearing in scheme I *alone* would give rise to oxalic acid.

## 2. Inhibition Studies

The presence in these molds of succinic dehydrogenase is demonstrated by their ability to give rise to oxalic acid from acetic and succinic acids. In the past it was observed that the action of this enzyme has been successfully inhibited by the use of malonic acid, by quinones, or by *p*-chloromercuribenzoate, for example. The effect of quinone in causing an irreversible inhibition of the system may be regarded as being due to oxidation of an—SH group contained in the activating protein in the sense of a thioether formation (8).



It was thought of interest to study the influence of quinone on the ability of the molds to produce oxalic acid from acetic acid. Inasmuch, however, as it has been observed that oxalic acid is produced by two pathways, it might be possible to inhibit completely the action of succinic dehydrogenase without affecting the formation of oxalic acid *via* the alternate path.

Experiments were set up using quinone in varying concentrations in the medium. Grown Meni mats were used. The medium employed contained 16 g. sodium acetate/l. The results of the experiments are presented in Fig. 2. It would appear that quinone has no measurable

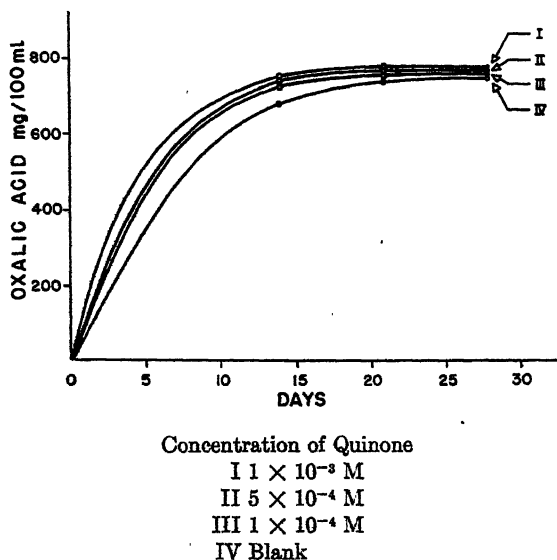


FIG. 2. Dissimilation of Sodium Acetate by *Merulius niveus* in the Presence of Quinone.

effect on the acetate  $\rightarrow$  oxalate transformation. This would not mean, however, that succinic dehydrogenase is not inhibited, since, as was stated above, oxalic acid could arise *via* the alternate pathway, acetic  $\rightarrow$  glycolic  $\rightarrow$  glyoxylic  $\rightarrow$  oxalic, although the other route is "blocked." Furthermore, the presence of  $-SH$  groups in enzymes of one cell system does not prove that such a system will possess  $-SH$  groups in others.

## COMMENTS

According to present concepts, the most significant constituents of wood are cellulose, lignin, and pentosans. Independent of the type of enzymatic attack on wood, that is, whether the destruction results in a brown rot or white rot, cellulose seems to be the main substrate of the enzyme action. Consequently, our interest has been directed toward an attempt to establish a so-called phase sequence of cellulose degradation by molds.

The results of our previous investigation of the action of these organisms on glucose, xylose, and cellulose suggested that they are perfect alcoholic fermenters, by-passing, however, the phase of phosphorylation.

Since the same metabolic products (ethyl alcohol and acetic acid) have been obtained from cellulose, glucose, or xylose by the action of these molds, we venture to postulate a mechanism for the breakdown of the cellulose fraction of wood by the action of these wood-destroying molds. On the basis of our findings, therefore, we herein present a picture of the fate of wood cellulose when attacked by these organisms:

Cellulose  $\rightarrow$  Glucose  $\rightarrow$  Ethyl Alcohol  $\rightarrow$  Acetic Acid  $\rightarrow$  Oxalic Acid

Barring the presence of an appropriate enzyme system and, since our molds are capable both of causing an alcoholic fermentation of *free* carbohydrates and of growing on wood, mainly with production of a brown rot, it would seem to us that our findings, in agreement with the considerations of Purves (9) or Kisser (10), indirectly support the postulation of the non-existence of a *chemical* linkage between cellulose and lignin in wood. On the other hand, the paramount existence of a strong dehydrogenating enzyme system in these molds clearly distinguishes them from such organisms as *Cl. aceticum* in which carbon dioxide is said to be used for the synthesis of acetic acid (11).

In this connection we must recall that the acetic acid formed is capable of serving as a switch-board for two chemically entirely different pathways of degradation ultimately giving rise to oxalic acid, and therefore we consider this course of reaction as a typical demonstration of a dualism in the enzymatic behavior of living organisms. Statements to the contrary (12) are, consequently, pointless. Such results should contribute to the better understanding of the paramount principle of self-regulation in living cells.

In conclusion, it should be pointed out that we do not think that each enzyme present in a system has to be or is ready to act at a certain moment. Our schemes as presented should serve only to visualize the simplest conceivable progress of a chemically symbolized phase sequence. However, we are fully aware of the fact that in complex and intact systems, such as our enzymatically unsaturated molds, this concept is not entirely satisfactory and, perhaps, prevents doing justice sufficiently to general biological requirements in the broader sense of totality.

#### ACKNOWLEDGMENT

This investigation was supported in part by grants from the Rockefeller Foundation and the Office of Naval Research. One of the authors (J.C.V.) wishes to thank Givaudan-Delawanna, Inc., New York, N. Y., for a fellowship.

#### SUMMARY

1. The action of the following wood-destroying molds on the following series of acids was studied: *Merulius confluens*, *Merulius niveus*, *Merulius tremellosus*, and *Fomes annosus* on acetic, glycolic, lactic, pyruvic, succinic, fumaric, malic, dimethylsuccinic and dimethylfumaric acids. With the exception of *Meco* the organisms were able to produce oxalic acid, present as a salt, from all the acids.

2. The organisms were able to utilize free oxalic acid but not when present as a salt.

3. The mechanism of acetate  $\rightarrow$  oxalate transformation proceeds along the following two pathways:

- (1) Acetic acid  $\rightarrow$  succinic acid  $\rightarrow$  fumaric acid  $\rightarrow$  malic acid  $\rightarrow$  oxalic acid.
- (2) Acetic acid  $\rightarrow$  glycolic acid  $\rightarrow$  glyoxylic acid  $\rightarrow$  oxalic acid.

4. Cellulose from wood is degraded according to the following phases through the actions of these molds: cellulose  $\rightarrow$  glucose  $\rightarrow$  ethyl alcohol  $\rightarrow$  acetic acid  $\rightarrow$  oxalic acid.

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On the Mechanism of Enzyme Action. XXX.  
The Formation of Methyl-*p*-Methoxycinnamate  
by the Action of *Lentinus lepideus* on  
Glucose and Xylose

F. F. Nord and James C. Vitucci

From the Department of Organic Chemistry,<sup>1</sup>  
Fordham University, New York 58, N. Y.

Received May 5, 1947

INTRODUCTION

The ability of certain molds to bring about the formation of complex synthetic products is evident when several specific compounds are considered. Thus, while, in general, mold products such as citric, fumaric, and oxalic acids, ethyl alcohol, *etc.*, represent simple breakdown entities, other compounds are more complex than the hexose or pentose molecule from which they may originate. As examples of synthetic products, citromycetin contains a benzopyrene nucleus while citrinin contains a benzene ring fused to another ring containing oxygen. More recently penicillin and streptomycin have been obtained as higher synthetic products of fungal metabolism.

A consideration of the metabolic activities of the higher *basidiomycetes* which grow on wood demonstrates the versatility of seemingly similar organisms. The fungi belonging to the class, members of which cause a brown rot in wood, bring about the preferential decomposition of cellulose and the associated pentosans. Although the wood is destroyed when attacked by this type of mold it is interesting that the metabolism of one member of the group differs from that of others despite the fact that the overall process, the decay of the wood, is the same.

Thus, although *Coniophora cerebella* (Cocer), *Merulius lacrymans* (Melac), *Polyporus vaporarius* (Povap), and *Lentinus lepideus* (Lelep) are classified as molds capable of causing a brown rot in wood, some difference in the enzymatic activities of the molds exists.

<sup>1</sup> Communication No. 55. Dedicated to Professor Carl Neuberg on his 70th birthday, July 29th, 1947.

Generally speaking, they are all acid formers. *Cocor* gives rise to acetic, formic and citric acids as well as traces of oxalic acid as degradation results of the action of the mold on wood (1). Falck (2) claimed to have identified free succinic and malic acids and combined oxalic and tartaric acids as metabolic products of the same organism. The formation of oxalic acid as a degradation product has also been demonstrated by Rabanus (3) as a result of the action of *Povap* on wood, and *Melac* too, has given rise to oxalic acid as a metabolite. Oxalic acid, then, appears to be the compound commonly obtained as a simple result of the degradation of wood by the action of molds causing a brown rot (4).

One member of this family, *Lelep*, differs from the others in that it is capable of synthesizing complex compounds when grown on white scots fir pine wood, and also when cultivated on a semi-synthetic glucose or xylose medium. By the action of *Lelep* on wood a crystalline substance was formed as well as an oily product having a strong, sweet, aromatic odor (5). The crystalline product was shown to be methyl-*p*-methoxycinnamate while the oily material was found to be a mixture of esters of cinnamic and anisic acids.

This, then, is an example of the formation of methylated products by fungi. That these products were the result of the action of the mold on wood was established, since it was demonstrated that they are absent from uninfected samples. In none of these cases, however, has any attempt been made to establish a route or mechanism for the formation of any of the above-mentioned compounds.

An interesting observation, during the study of the enzymic activities of a number of wood-destroying molds, was the formation of a well-defined crystalline substance as a metabolic product when *Lelep* was grown on a semi-synthetic medium with glucose and xylose as the sole carbon source. The compound proved to be identical with that obtained by the action of the organism on wood, namely methyl-*p*-methoxycinnamate.

#### EXPERIMENTAL

The crystalline compound was obtained when *Lelep*, a culture of which was received through the courtesy of Dr. William J. Robbins of the N. Y. Botanical Gardens, was grown on the following medium:

Carbohydrate.....	20 g.
$\text{KH}_2\text{PO}_4$ .....	1.5 g.
Neopeptone.....	1 g.
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ .....	0.5 g.
Thiamine-HCl.....	2 mg.
Tap water to .....	1 liter

Stock cultures of the organism were maintained on the above medium supplemented with 20 g. of agar. Transfers were made every two weeks and growth progressed in the dark at 27°C.

The nutrient medium was sterilized by steam for 20 minutes at 15 lbs. pressure and inoculations made by adding a uniform spore-mycelial suspension. Fifty ml. of this suspension was added to 3 l. Fernbach flasks containing 1 l. of the above medium.

### Isolation

Two liters of the medium were made up and distributed as 1-liter lots in 3-liter Fernbach containers. After sterilization, each flask was sown with the spore-mycelial suspension and incubated for a period of 40 days. At the end of this period the medium containing the crystalline product and mycelium were filtered, washed with cold water and dried. The dried mycelium was ground and thoroughly extracted overnight with 300 ml. of carbon tetrachloride. On evaporating the solvent, 100 mg. of crude product was obtained. Purification was effected by sublimation *in vacuo*. A white crystalline product was obtained, m.p. 88°C. A mixed melting point taken with an authentic sample of methyl-*p*-methoxycinnamate showed no depression. Data representing a typical experiment are given below:

Incubation period.....	40 days
Initial glucose concentration.....	18.45 g./l.
Final glucose concentration.....	11.52 g./l.
Mycelium weight.....	4.7 g./2 l.
Yield of crude product.....	100 mg./l.
Analytical data: Calculated for $C_{11}H_{12}O_3$ :	C, 68.74%; H, 6.30%
Found:	C, 68.90%; H, 6.18%

### Characterization

*Oxidation of Compound.* One gram of the purified ester was dissolved in 100 ml. of pure acetone and about 3 g. of finely powdered  $KMnO_4$  added in small portions with shaking and cooling under the water tap. The solution was then filtered and the precipitate washed with acetone and dried. The dried material was extracted with water and the clear, colorless filtrate acidified with HCl. The crystals were filtered, washed with cold water and dried. One-half gram of the crude material was recrystallized from water, m.p. 183°C.

Analytical data for anisic acid  $C_8H_8O_3$ :

Calculated:	C, 63.17%; H, 5.30%
Found:	C, 63.15%; H, 5.34%

*Saponification of Compound.* Hydrolysis of the compound was effected by heating 1 g. with 25 ml. of *N* methyl alcoholic KOH for 30 minutes under reflux. The solid first dissolved and then crystals of



the potassium salt separated out. These were dissolved by addition of about 50 ml. of water and the mixture acidified with HCl. The precipitated acid was filtered and dried *in vacuo*, and 0.9 g. of crude material was obtained. The product was recrystallized from benzene, m.p. 173°C.

Analytical data for *p*-methoxycinnamic acid,  $C_{10}H_{10}O_3$ :

Calculated: C, 67.41%; H, 5.66%

Found: C, 67.40%; H, 5.43%

#### COMMENT

The interesting observation that Lelep acts on wood as well as on glucose or xylose to give rise to methyl-*p*-methoxycinnamate as a metabolic product, corroborates the theory of the degradation of the cellulose of wood *via* an introductory hydrolysis when it is decomposed by microorganisms. According to this concept, an exoenzyme secreted by the microorganism causes the hydrolysis of the cellulose molecule. This gives rise to glucose molecules which are subsequently dissimilated by the enzyme in the cells of the organism, resulting in a variety of end products, depending on the type of organisms causing the dissimilation. Since it has been demonstrated that Lelep produces methyl-*p*-methoxycinnamate from glucose as well as from wood, it is only through a hydrolytic split of the cellulose that glucose can arise as a primary dissimilation product. Then by further action of the organism the synthetic product is formed from the free carbon compound.

The significant observation, however, that the action of Lelep on xylose gives rise to the same compound prompts, of course, a consideration as to the mechanism of its formation. It would be simple in the case of glucose alone to assume that perhaps a cyclization of a straight-chain compound, similar to the assumed formation of inositol (6), is the basic step of its origin. Two observations, however, induce us, at least for the time being, to disregard this consideration. First, that the pentose, xylose, when acted upon by the same organism, also gives rise to the same compound and, second, the ethyl alcohol obtainable from both carbohydrates is rapidly dehydrogenated, prompting the consideration of the transitory formation of a C-2 compound as a second switchboard in this multipath enzymic synthesis. The latter possibility is induced by a rapid dehydrogenation of ethyl alcohol when it served as a sole carbon source of Lelep in the presence of resazurin.

Attempts to dehydrogenate ethylene glycol which, in the case of *Fusarium lini* Bolley, readily gave rise to the formation of glycolaldehyde (7), which could serve as a key compound in this enzymatic synthesis, were, however, of no avail.

This investigation was supported in part by grants from the Rockefeller Foundation and the Office of Naval Research. One of the authors (J.C.V.) wishes to thank Givaudan-Delawanna, Inc., New York, N. Y., for a fellowship.

### SUMMARY

1. The wood-destroying mold, *Lentinus lepideus* produces, as a metabolite, methyl-*p*-methoxycinnamate from glucose and xylose.

2. The observation of the formation of methyl-*p*-methoxycinnamate by the action of *Lentinus lepideus* on glucose, xylose, and wood supports the concept that the phase sequence of the decomposition of the cellulose of wood is initiated by an introductory hydrolysis of cellulose caused by the microorganism.

3. A preliminary consideration as to the origin of methyl-*p*-methoxycinnamate from glucose and xylose is presented.

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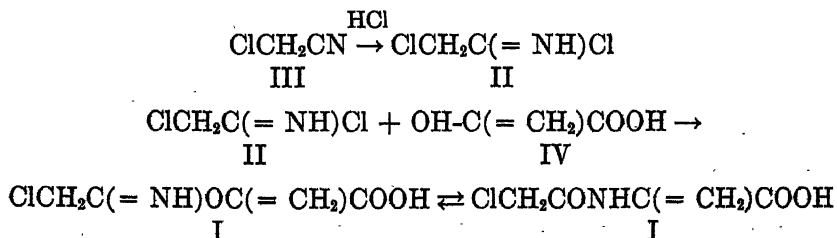


## A New Synthesis of Chloroacetyldehydroalanine

Bergmann and Grafe (1) reported that a mixture of chloroacetamide and pyruvic acid heated *in vacuo* under a reflux condenser gave about a 20% yield of chloroacetyldehydroalanine (N-chloroacetyl- $\alpha$ -amino-acrylic acid). We have found this method unreliable, achieving only two successful preparations out of about 20 attempts.

A simple, reproducible, and nearly quantitative synthesis of this peptide is as follows: 7.5 g. (1 mole) of redistilled chloroacetonitrile and 11.4 g. (1.3 mole) of redistilled pyruvic acid are mixed, chilled in an ice bath, and the mixture saturated with dry HCl gas. The reaction is strongly exothermic. After standing for a few hours in the cold, the mixture turns into a solid mass of crystals of chloroacetyldehydroalanine, which is washed on the filter with several portions of dry ethyl ether, and dried in air. The yield of nearly pure peptide is invariably 80–90% of the theory. N found 8.7%, N calculated 8.6%. The compound has a melting point of 159°C., and possesses the characteristic absorption spectrum in the ultraviolet (2). A single crystallization from acetone gives a product with a melting point of 162°C. uncorr. N found 8.6%, N calculated 8.6%. Bergmann and Grafe reported a melting point for their preparation of 163–165°C. corr.

No reaction occurs in the absence of the HCl gas. It is assumed that the synthesis of the peptide (I) is achieved through the interaction of the imino chloride derivative (II) of chloroacetonitrile (III) with the enolic form of pyruvic acid (IV):



The method is applicable to other nitriles and keto acids, and permits the ready synthesis of this class of peptides which have been found to be substrates for active and wide-spread enzyme systems in animal tissues (2, 3).

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National Cancer Institute,  
National Institute of Health,  
Bethesda, Maryland.  
April 29, 1947

VINCENT E. PRICE  
JESSE P. GREENSTEIN

### A Note on the Preparation of Crystalline Soy Bean Lipoxidase

Lipoxidase, an enzyme capable of promoting the oxidation of linoleic acid and other unsaturated fatty acids by atmospheric oxygen, has been isolated in pure form from soy beans. The spectrophotometric method of assay for lipoxidase activity developed in this laboratory (1) has been used for all activity determinations in this study.

The method of separation of lipoxidase is briefly as follows: Defatted soy meal was extracted with acetate buffer at pH 4.5, where the enzyme is soluble but less total material dissolves than at neutrality. The extract was brought to pH 6.75 and barium acetate, basic lead acetate, and acetone were added to precipitate inactive material. The enzyme was precipitated from the solution by ammonium sulfate in a concentration of 400 g./kg. solution, and the precipitate was redissolved in a minimum of water and heated to 63°C. for 5 minutes to precipitate additional inactive material. The mother liquor was fractionated with ammonium sulfate, then with alcohol in the cold, and then again with ammonium sulfate. The most active fraction was subjected to electrophoresis at pH 6.0 which separated the enzyme from the bulk of the remaining impurities. Crystallization was achieved by slow dialysis of a concentrated enzyme solution against increasing concentration of ammonium sulfate. The crystals (see Fig. 1), colorless microscopic

plates, were slightly more active than the mother liquor, and their activity was equal to the value calculated from the activities and electrophoretic patterns of impure samples.

The crystalline preparation was found to be homogeneous as judged from sedimentation and diffusion measurements made at the Physical-Chemical Institute at Upsala University by courtesy of Dr. K. O. Pedersen. The molecular weight lies between 90,000 and 100,000, and determination of iron on a nearly pure sample indicated that any iron present must be present as an impurity. This is in agreement with the observations that fluoride, cyanide, pyrophosphate and azide do not inhibit the enzyme action even in high concentrations.



FIG. 1. Lipoxidase Crystals from Ammonium Sulfate (1200 $\times$ )

The activity of the enzyme has been found to be 460 units (1)/density unit at 2800 Å, or approximately 780 units/mg. Assuming a molecular weight of 94,000 and a molecular extinction coefficient of 32,000 for the conjugated hydroperoxidic reaction product, the activity index of the lipoxidase is 290 moles of peroxide produced per molecule lipoxidase per second at 20°C. at optimum substrate concentration. The pure enzyme represents approximately a 115-fold purification from the crude buffer extract of the soy bean meal.

The details of the method of preparation of crystalline lipoxidase and a discussion of its properties will be presented in detail at a later date in the *Acta Chemica Scandinavica*.

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*Biokemiska Avdelningen,  
Medicinska Nobelinstitutet,  
Stockholm, Sweden.  
May 20, 1947.*

HUGO THEORELL  
RALPH T. HOLMAN \*  
ÅKE ÅKESON

\* National Research Council Fellow.

These two papers, dedicated to Dr. Neuberg's 70th birthday, arrived too late to be included in this issue and will appear in Volume 14, Number 3.

THE CHEMISTRY OF INFECTIOUS DISEASES: VIII. Partial Amino Acid Composition of Purified Dog Serum Albumins before and during Type I Pneumococcal Pneumonia, and IX. Partial Amino Acid Composition of Salt Fractionated Dog Serum Globulins before and during Type I Pneumococcal Pneumonia," by Bruno Vassel, Ruth Partridge, and M. L. Crossley.

# Chlorophyll Fluorescence and Photosynthesis in Algae, Leaves and Chloroplasts

Yen G. Shiau and James Franck

*From the Department of Chemistry (Fels Fund), University of Chicago*

Received March 25, 1947

## INTRODUCTION

The chlorophyll protein complexes of green plant cells have the property of fluorescing. Kautsky and co-workers (18, 19) were the first to observe that the fluorescence intensity varies in a typical way during the induction period of photosynthesis, and to recognize that these transient intensity anomalies may be of importance for an interpretation of the photochemical steps of photosynthesis. Franck and co-workers (6, 7, 8) and McAlister and co-workers (24) extended our knowledge of this field in the United States. Much important work was done in Holland by Wassink, Katz and co-workers (33, 34), who not only made studies of the chlorophyll fluorescence of green plants, but extended their observations to the fluorescence in purple bacteria and diatoms. There is, in general, an agreement between the experimental results of these different authors insofar as their observations overlap, but practically no agreement has been reached regarding the theoretical interpretations which, at least in their basic principles, must be the same for green plants as for purple bacteria and diatoms. The present paper is devoted to observations and interpretations of the phenomena in green plants. The principles used can be applied also for an explanation of the observations with photosynthesizing bacteria and diatoms that will be shown on another occasion.

The main result gained by observations with green plants is the following. The yield of fluorescence light is of the order of magnitude of 0.1% if the plant is engaged in photosynthesis with optimal quantum yield but rises if the photosynthetic activity is inhibited by internal or external conditions. But even if narcotic, surface-active inhibitors are used, which cover the surfaces of the chloroplasts to such a degree that practically all photochemical activity is stopped, the fluorescence intensity of the chlorophyll becomes only about 2-3 times higher than normal. Many attempts have



been made—only partially successful—to interpret these and related phenomena by the assumption that quenching of chlorophyll fluorescence by contact with photochemically sensitive molecules was entirely identical with quenching of fluorescence of monatomic or diatomic gases. According to Franck and Livingston (10), this assumption is not true. In polyatomic dyestuffs, like chlorophyll, the fluorescence yield will be very small, even in the absence of photochemically sensitive substances if the lifetime of the excited state is very much shortened by a spontaneous "internal conversion" of the excitation energy stored in the electronic system into atomic oscillation energy.

As a consequence of the internal conversion, either a shift of a hydrogen atom in the dye molecule may occur (Franck and Livingston) or the electronic system of the dye molecule may be transferred into a metastable "triplet" state (G. N. Lewis and co-workers (22)). In either case, an energy-rich, excited state of the dye is produced which has a longer lifetime than the fluorescent state of the dye. If photosensitive hydrogen acceptors are in contact with the dye during the lifetime of its energy-rich state, a hydrogen atom can be transferred from the dye to the acceptor molecule as experiments with chlorophyll in organic solvents have indicated. If, on the other hand, no hydrogen acceptor is present, the energy-rich dye molecule returns to its normal state by converting its stored energy into heat or it may emit the energy as phosphorescence light.<sup>1</sup>

In plants the process is supposed to be principally the same as *in vitro*, except that the dissolved chlorophyll molecules are replaced by chlorophyll protein complexes in plants. Whether the chlorophyll itself or the protein acts in this case as the primary hydrogen donor is open to discussion. The shifting of a hydrogen atom does not occur immediately at the moment the dye is excited by light absorption, but after a time lapse in which all the atoms in the complex attain such positions, relative to each other, as are favorable for the internal conversion or external transfer of the hydrogen atom. The time lapse needed to attain such positions depends upon the structure of the complex and upon the mobility of its atoms. If the mobility of the atoms in the complex is reduced by high viscosity of a solvent or by strong adsorption to surfaces, the time lapse becomes greater and the fluorescence intensity rises correspondingly. Contact with hydrogen acceptors may either strengthen or weaken the fluorescence intensity, depending on whether the influence of the reduction of the atomic mobility by their attachment of the hydrogen acceptor prevails, or whether the new position offered by the hydrogen acceptor is so much more easily

<sup>1</sup> Phosphorescence of chlorophyll dissolved in some organic solvents is observed, but experiments with plants carried out under conditions favorable for the observation of chlorophyll phosphorescence, are thus far lacking. (Kautsky, H., *Ber.* 68, 152, 1935; Calvin, M. and Dorough, G. D., *Science* 105, 433, 1947.)

accessible to the hydrogen atom than the position in the original complex that this factor overbalances the first-mentioned influence.

The fact that a chemically inactive narcotic which is strongly adsorbed at the surface of the chlorophyll protein complex considerably strengthens the fluorescence is evidently in agreement with the theoretical expectation, but the observation that the intensity also rises in green plants if the  $\text{CO}_2$  uptake becomes the limiting factor, can be interpreted in two ways. Either the chlorophyll complex fluoresces more strongly when denuded or the photosynthetically reducible substances, *i.e.*, photosensitive hydrogen acceptor, may be replaced by other narcotic molecules around the chlorophyll. Indeed, under  $\text{CO}_2$  limitations (if special methods, to be described in this paper, are not used) a narcotic substance is formed and starts to cover the chlorophyll surface. This substance can be called a natural narcotic to differentiate it from the surface-active substances artificially administered to the plants. If there is a direct influence of the contact of the reducible substances to be photosensitized on the fluorescence intensity, it will be masked by the influence of the strongly absorbed narcotic.

The production of surface-active substances as a result of the photochemical processes occurring in the plants was first postulated by Franck, French and Puck (7) to explain the phenomena of the normal induction period of photosynthesis, especially the systematic connection between temporary rises of the fluorescence yield with depressions of the photosynthetic rates. The interpretation of the induction phenomena is briefly the following: During a dark period preceding the illumination, an enzyme engaged in the liberation of oxygen from the peroxides formed by photosynthesis becomes inactivated; its reactivation is achieved upon illumination with the help of a photosynthetic product. At the start of the illumination the peroxides, because of the inactivity of the enzyme, are not removed quickly enough to prevent oxidation of organic matter. Preferentially easily oxidizable substances like reducing sugars are attacked. The first oxidation product of the carbohydrates—supposed to be acids—settles down on the chlorophyll surface, thereby quickly raising the fluorescence yield while reducing the photosynthetic production of the peroxides to a level which the available enzyme can handle. During the following period, lasting longer than the first one, the narcotic layer is gradually removed by further oxidation (mostly respiration), while the enzyme is gradually reactiv-

vated and, correspondingly, the fluorescence intensity goes down and the photosynthetic rate rises to the normal value.

In the meantime more was learned by Franck, Pringsheim and Lad (11) about the deactivation of the oxygen-liberating enzyme from the studies of the photosynthetic oxygen production of algae under strict anaerobic conditions. In dense cultures of algae this treatment causes almost total deactivation of the oxygen-liberating enzyme and the inactivity persists during illumination if the small amount of oxygen evolved is carried away quickly enough by a stream of oxygen-free gas. If, however, the oxygen is permitted to accumulate, the activity of the enzyme is gradually restored. In very dilute algae suspensions the poisoning effect is so small that the few algae present produce, under anaerobic conditions, almost as much oxygen as under aerobic conditions. Anaerobic metabolism either produces a water-soluble poison of the enzyme which permeates all the cell membranes at a rate which is much higher than the rate of poison production by anaerobic metabolism, or, if the rate of poison production does not depend upon the presence of air, then it is the permeability of the membranes which increases under anaerobic conditions.

The fact that the densely packed cells of leaves show much more pronounced induction phenomena in air than algal suspensions with much smaller cell concentration, suggests that the poisoning of the oxygen-liberating enzyme in the dark is caused by the same metabolic product which causes the much more pronounced poisoning effect under anaerobic conditions.

In the same category belongs, in our opinion, the fact observed by Pratt (29) that dense cultures of algae which have remained for a long time in the same nutrient solution stop photosynthesizing and growing under the influence of a water-soluble, membrane-penetrating poison. We are inclined to believe that the limitations are imposed by the same poison which inhibits the oxygen evolution and indirectly the photosynthetic activity.<sup>2</sup>

When plants are strongly irradiated, while  $\text{CO}_2$  is limiting, photooxidation involving molecular oxygen occurs (7). The suggestion by Franck and Gaffron (8) is that this method of oxidation will also produce the surface-covering substance. Indeed, it is observed by McAlister that the fluorescence yield of leaves rises with light intensities in air in the region where light saturation is almost reached while, if irradiated in an atmosphere of nitrogen containing only a few tenths

<sup>2</sup> Whether the antibiotic substance extracted by Spoehr and co-workers (32) from the algae themselves may be identical with this enzymatic poison is uncertain. It is, furthermore, too early to draw any conclusions as to where the enzymatic poison is made, either inside the cells in regions separated from the chlorophyll by membranes which protect the enzyme, or at the outer surface of the cells.

of 1% of oxygen, they still have a constant fluorescence yield. More observations connected with this problem will be found in the present paper. It may be mentioned that prolonged, strict anaerobic condition in oxygen-free nitrogen or hydrogen produces in algae a surface-covering narcotic of another type by fermentation. Observations of the photosynthetic activity under anaerobic conditions (11) offered indirect evidence of this effect, and it is confirmed by our fluorescence measurements, which will be discussed in greater detail in the present paper.

The experiments in this paper were carried out to supplement our knowledge of the chlorophyll fluorescence in green plants. They especially serve to test the point of view presented in the introduction that the production of surface-active narcotic is used by the plant as a means of checking its photochemical activity in order to prevent damaging photoreactions, such as excessive oxidations.

#### APPARATUS AND METHODS

The apparatus used in measuring the time course of fluorescence intensity variations of the plant material is primarily the same as that described by Franck, French and Puck. It is diagrammed as in Fig. 1. The exciting light from a 500-watt tungsten filament projection lamp, B, equipped with a constant voltage regulator, was restricted to wavelengths between 4,000 and 6,000 Å by a system of filters, C, E, F, G, which are 5 cm. H<sub>2</sub>O, 5 mm. Corning 3966, 2 mm. Corning 3850 and 5 cm. 10% CuSO<sub>4</sub> solution. This light was focussed by a system of condenser lenses, D, through a camera

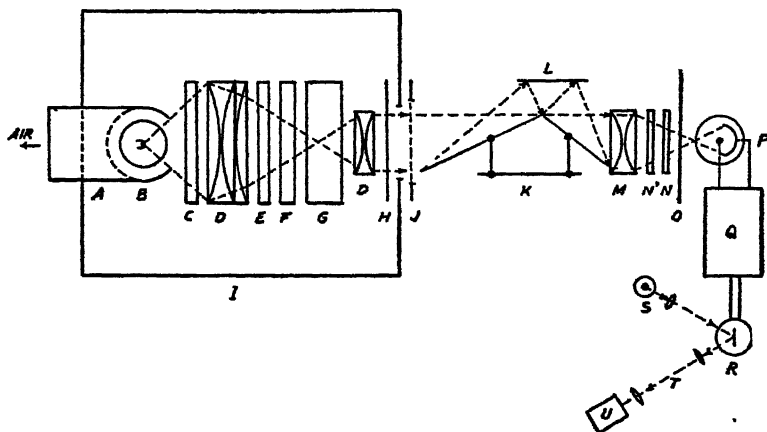


FIG. 1. The apparatus used in measuring and recording the time course of fluorescence intensity variations of the plant material.

shutter, H, to form a uniformly illuminated spot about 5 cm. in diameter on the leaf or suspension of algae and chloroplasts at L. K is a system of adjustable mirror reflectors. The intensity of this illumination was about  $3.0 \times 10^4$  erg/cm.<sup>2</sup>/sec. as measured by a thermopile-galvanometer system which had been calibrated with a standard lamp. This intensity suffices to give saturation of photosynthesis. A set of copper wire screens of different mesh, J, was used to intercept the incidental beam to obtain a wide range of weaker light intensities. The fluorescence light of the plant material was then focussed on a photo cell, RCA 922, P, through 2 mm. red filters, Corning 2404, N, and 3966, N'. This arrangement insured that little of the exciting light reached the photo cell while a high percentage of the fluorescence light was transmitted to it.

A rotating sector, O, placed just before the photo cell interrupted the fluorescence light beam 240×/sec. The resulting photoelectric current was amplified by a 5-stage A.C. amplifier, Q, and then fed to a vibration galvanometer, R, the mirror of which made a complete oscillation for each pulse of light on the photo cell. A beam of light, T, was reflected by the mirror through a system of cylindrical lenses to the photographic recording paper, U, 2 meters away. The vibrations, proportional in amplitude to the intensity of the fluorescence light, were continuously recorded on the moving photographic paper, the speed of which could be varied over a wide range. This galvanometer had a period of 0.004 second, requiring about five vibrations, or 0.02 sec., to respond fully to any sudden and great change of light intensity.

The gas line, diagrammed in Fig. 2, was used to pass the desired gas through the suspension of algae or chloroplasts. To measure their fluorescence intensity under continuously anaerobic conditions, the gas line was first evacuated and then filled with N<sub>2</sub> or H<sub>2</sub> rendered oxygen-free by passing over hot, finely-divided Cu, adsorbed in Fuller's earth, in column B. This method was devised by Meyer and Ronge, (26), and yields gas in which the oxygen partial pressure is less than  $10^{-6}$  mm. N<sub>2</sub> is then led through the liquid nitrogen trap C and either through a saturated solution of NaHCO<sub>3</sub>, in vessel D, to be enriched with CO<sub>2</sub>, or through a saturated solution of Ba(OH)<sub>2</sub> in vessel F, to be freed from CO<sub>2</sub>. It is then passed in many fine bubbles through a fritted glass filter through the suspension of algae or chloroplasts in vessel G, as described in (11). This vessel is immersed in distilled water at the desired temperature in an unsilvered Dewar flask. As the fluorescence intensity varied to a certain extent with the rate of flow, the latter was kept constant by good valves and accurately measured by a sensitive flowmeter I. To test for any O<sub>2</sub>, this line could be coupled at H<sub>2</sub> to the apparatus of Pollack *et al.* (28), which is capable of measuring O<sub>2</sub> of very low concentration or, more simply, to a Thunberg tube containing methylene white.

To measure fluorescence intensity under aerobic conditions, air was slowly drawn through the stopcock D<sub>1</sub>, vessels D or F, flowmeter I and out through the water pump. The pressure above the suspension must be maintained lower than the pressure below the suspension of plant material to prevent loss of plant material. Fluctuations in the water pump were minimized by a manostat J and the rate of flow regulated by the grooved stopcock J<sub>1</sub>. Gas mixtures of N<sub>2</sub>, O<sub>2</sub> and CO<sub>2</sub> in desired compositions were prepared in the gasometer E, and then drawn out and flushed through the suspension as described above. The surfaces of Hg in the 2 bulbs were kept at the same level to give a constant rate of flow.

The two pure strains of the unicellular green algae used were *Chlorella pyrenoidosa* and *Scenedesmus obliquus* (strain D<sub>3</sub>). They were inoculated and grown in this laboratory in the usual manner, as described by Gaffron and Ruben (15). The culture was always illuminated for about two hours under growing conditions just before use and was then diluted with distilled water to a concentration of 0.05% by volume. Ten cc. of this suspension were used in all experiments reported in this paper. In general, a given culture was used for about 10 days.

Chloroplasts isolated from the leaves of *Tobacco Havana 38* grown in the greenhouse of the Department of Botany and occasionally from spinach leaves obtained in the market were used. The procedures for the isolation of the chloroplasts were

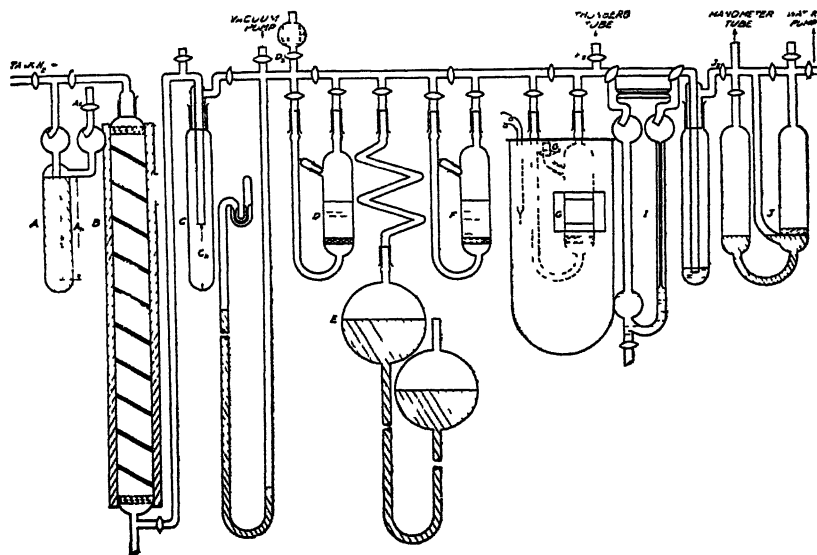


FIG. 2. The gas line used in flushing the desired gas through the suspension of the plant materials.

described by French and co-workers (12) and Franck (6). Care was taken to prepare the chloroplast suspension in the cold room in the dark to obtain the best activity. The specific values of speed and time used in the fractional centrifugation depended on the size and condition of the leaves and frequent microscopic examinations of the contents of the suspension helped to isolate the chloroplasts properly.

*Tobacco Havana 58* was employed in the experiments with leaves. The pressed leaves were prepared by pressing a circular disk of the leaf parenchyma between two glass slides until the leaves appeared translucent but without any noticeable loss of the plant sap. The leaf was held firmly in a chamber provided with a glass window and gas inlets and outlets through which air containing 4% CO<sub>2</sub> and water vapor was passed.

## RESULTS AND DISCUSSION

## A. FLUORESCENCE MEASUREMENTS OF ALGAE

Fluorescence intensity variations during the induction period were carefully measured under various conditions by Wassink and Katz (33). However, it seemed necessary to supplement these observations with new ones to clarify various points not covered by the work of these authors. Our apparatus, having a much smaller inertia than theirs, could measure more accurately the starting point of fluorescence intensity at the very beginning of the illumination period. This is of importance for the solution of such questions as whether, and under what conditions, narcotic surface layers are formed in the preceding dark period. Also, observations of the fluorescence with *Scenedesmus* and *Chlorella* under strict anaerobic conditions became necessary. The influence on the fluorescence intensity of alkali and of substances other than  $\text{CO}_2$ , found recently to be reducible in plants, had to be studied and also more observations on the influence of the previous history of the algae culture on the induction phenomena became desirable.

## 1. Fluorescence Time Curves

The curves presented here are reproduced from the continuous recording of the fluorescence intensities *versus* time curves as done in (7). Fig. 3a shows a fluorescence time curve of a culture of *Chlorella* in nitrogen and in air (both gases were enriched with 2% carbon dioxide) at 25°C., and  $3.0 \times 10^4$  erg cm.<sup>2</sup> sec. incident light intensity, and Fig. 3b shows that of *Scenedesmus* under the same conditions. Figs. 4a and b show the corresponding initial fluorescence rise curves of *Chlorella* and *Scenedesmus*, respectively, as registered at the maximum speed of the recording camera, using a high-speed camera shutter.

There is no essential difference between *Scenedesmus* and *Chlorella* as regards the effect of nitrogen on their fluorescence behavior. The initial fluorescence intensity in nitrogen is considerably higher and is followed by a less extensive and slower rise to a maximum than in air. This maximum lies higher and is followed by a slower decay in nitrogen than in air. The steady state fluorescence intensity reached in 2-3 minutes is higher in nitrogen than in air if nitrogen is continuously being flushed through the suspension. After the steady state has been reached during an illumination under strict anaerobic conditions, a dark period

of about 15 minutes is necessary for the recovery of this induction phenomenon so that the initial fluorescence intensity again becomes high upon the succeeding illumination

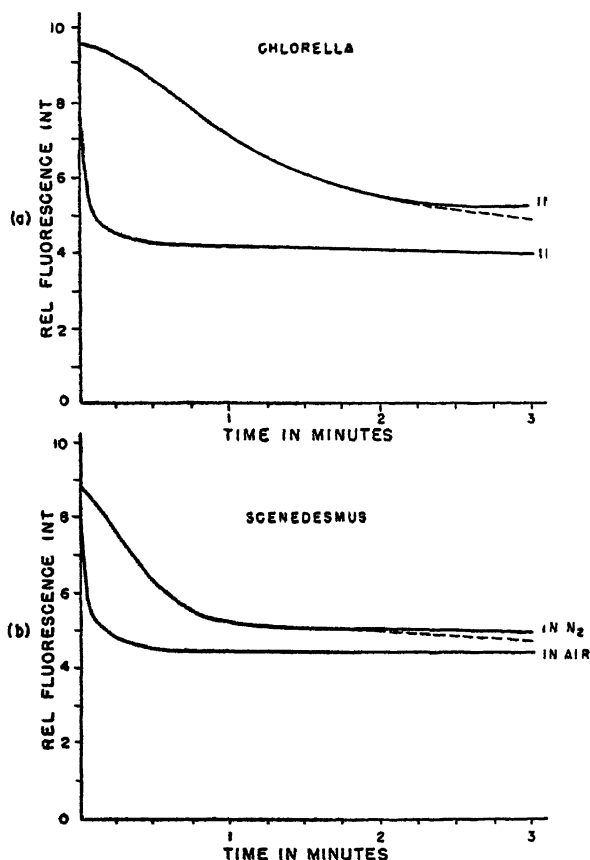


FIG. 3. Fluorescence time curves of (a) *Chlorella* and (b) *Scenedesmus*, 25°C. and  $3.0 \times 10^4$  erg/cm.<sup>2</sup>/sec. incident light intensity, showing the induction phenomena. Relative fluorescence intensities are expressed in arbitrary units which differ for (a) and (b).

When oxygen from photosynthesis is allowed to accumulate in the fluorescence vessel by shutting off the nitrogen flow, the difference in the fluorescence intensity in air and in nitrogen after 3 minutes of ir-



radiation is smaller, as shown by the dotted lines in Fig. 3, and vanishes as more oxygen is accumulated in about one hour. If the suspension is not stirred by the nitrogen bubbles flushing through the algae, it starts to settle down on the glass filter and thus affects the fluorescence in-

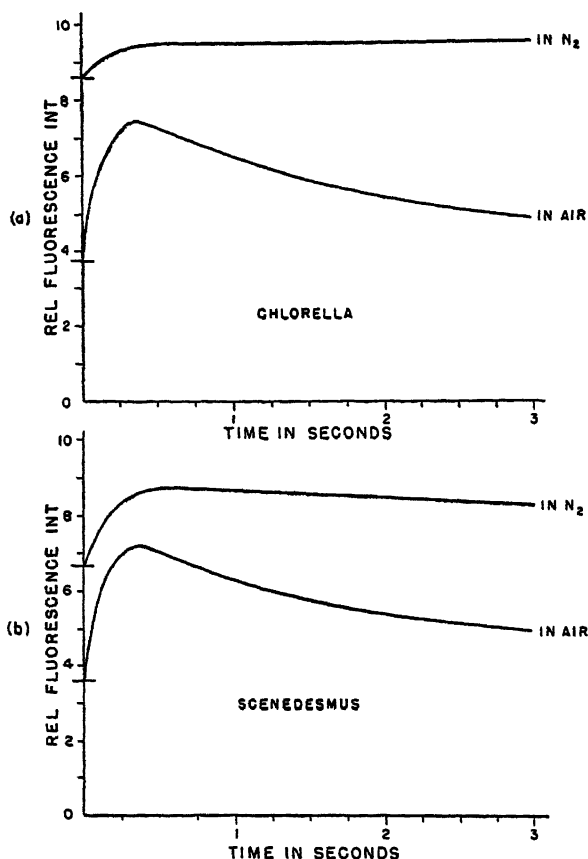


FIG. 4. Fluorescence time curves of (a) *Chlorella* and (b) *Scenedesmus* at 25°C. and  $3.0 \times 10^4$  erg/cm.<sup>2</sup>/sec., showing the initial rises.

tensity. A correction factor of about 10% for this effect had to be applied. This factor was determined by observing the difference between the steady fluorescence intensity of the suspension when air was flushed through and when it was shut off.

In the case of *Scenedesmus*, the nitrogen effect of increasing the initial fluorescence intensity and in decreasing the subsequent initial rise is generally observed to be more pronounced than in *Chlorella* under the same cultural and experimental conditions. However, the nitrogen effect in decreasing the steady state value is less pronounced in *Scenedesmus* than in *Chlorella*, as a rule.

The fluorescence behavior of algae in hydrogen showed no essential difference from that of nitrogen on the basis of several observations made with hydrogen.

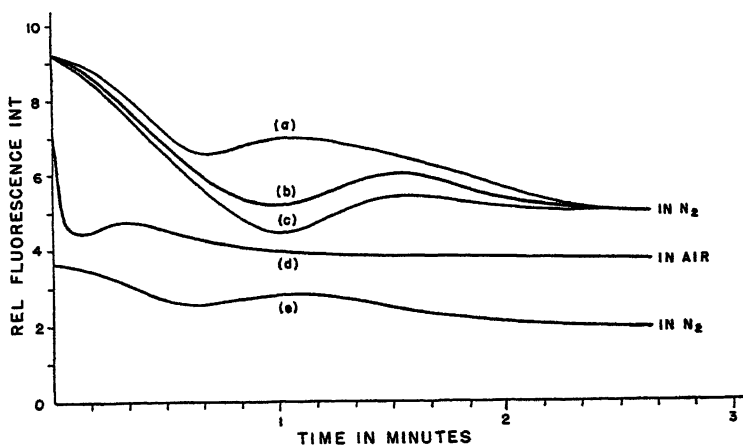


FIG. 5. Fluorescence time curves of *Chlorella* at 24°C., showing the secondary induction under different conditions.

	Time in darkness:		Light intensities:
	Hour	Gas	erg/cm. <sup>2</sup> /sec.
(a)	0.5	N <sub>2</sub>	$3.0 \times 10^4$
(b)	1.0	N <sub>2</sub>	$3.0 \times 10^4$
(c)	2.0	N <sub>2</sub>	$3.0 \times 10^4$
(d)	0.5	Air	$3.0 \times 10^4$
(e)	0.5	N <sub>2</sub>	$2.2 \times 10^4$

## 2. Secondary Induction Phenomena

Fig. 5 shows typical fluorescence-time curves which resulted frequently with *Chlorella* and, less frequently, with *Scenedesmus* at room temperature. The course of fluorescence decay is interrupted by a transient rise and a secondary maximum superimposed on the general decay. This phenomenon was always present in the observations of

Wassink and Katz with *Chlorella*. The corresponding secondary induction in the rate-time curve of oxygen production by algae anaerobically was also frequently observed by Franck *et al.*, who found that the secondary induction is more pronounced if CO<sub>2</sub> uptake by algae is limited (11) or if the cultural age of algae is old (7). It was also observed in the aerobic measurements of the time course of photosynthetic uptake of carbon dioxide by McAlister and Meyer (24) and by Aufdemgarten (2).

The secondary induction is more pronounced and occurs later after the beginning of illumination in nitrogen than in air. The extent, duration, and time of occurrence of this secondary induction increases with the period of anaerobiosis in darkness. The three curves, a, b, and c, which were measured in nitrogen at the usual light intensity of  $3.0 \times 10^4$  erg/cm.<sup>2</sup>/sec. after anaerobic periods of 0.5, 1 and 2 hours, respectively, show the effect of prolonged anaerobiosis. Curve e, which was measured at a lower light intensity of  $2.2 \times 10^4$  erg/cm.<sup>2</sup>/sec. after 0.5 hour of anaerobiosis, as compared with curve a which was measured at an intensity of  $3.0 \times 10^4$  erg/cm.<sup>2</sup>/sec., shows the effect of light intensity. Curve d, which was measured in air, shows the influence of air on the secondary induction when compared to Curve a.

### 3. Influences of the Cultural Conditions

It was observed that with some very young and dilute cultures of algae, both *Scenedesmus* and *Chlorella*, no induction phenomena occurred at all. The fluorescence intensity of these cultures remains constant from the very beginning of illumination. However, after long anaerobic incubation, their fluorescence shows the normal behavior in nitrogen (as in Fig. 3). These young cultures under consideration were examined and found to be very healthy, and to grow, multiply and photosynthesize rapidly.

Furthermore, it was observed that some very old cultures have extra long induction periods which often last as long as 5 minutes in air as compared to the usual one-minute period. Also, these cultures often have a very long induction phenomenon in nitrogen and the fluorescence intensity remains at the high initial value for over 5 minutes.

Between these two extremes of no induction and an extra long one with young and old cultures, respectively, we found varying degrees of induction loss. As may be expected, young cultures were, in general,

found to have shorter induction periods and less induction loss than the old ones. When the medium of suspension of such old and new cultures were interchanged by means of centrifugation and resuspension, no effect was observed upon the old cultures, and they behaved just as before, 24 hours after the interchange of the suspension media. However, the young ones, after being suspended 24 hours in the media which previously contained old cultures, often, though not always, began to show more induction loss and longer induction periods, depending upon internal cultural conditions.

#### 4. Steady State Fluorescence

The steady state fluorescence of algae *Chlorella* was previously studied by Wassink *et al.* (34). From their observations, they concluded that the curve of fluorescence intensity *versus* the incident light intensity is a straight line well into the region of light saturation of photosynthesis. The steady state fluorescence of *Chlorella* and *Scenedesmus* under aerobic and anaerobic conditions was also studied by us, with the result that, with our cultures of algae, the fluorescence yield increases at higher light intensities.

Figs. 6a and b show the curves of steady state fluorescence intensity *versus* the incident light intensity at 25°C. for *Chlorella* and *Scenedesmus*, respectively. The steady state fluorescence is usually attained after 3-4 minutes of illumination at room temperature. To obtain the curves reported here, the steady state fluorescence at the lowest intensity used was first measured. The intensity was then increased stepwise to measure the successive points after about 1-2 minutes of illumination, which proved sufficient to attain the new steady state. The procedure was then reversed by decreasing the intensity stepwise from the highest intensity used. The two measurements were found to check very closely.

The curves in Fig. 6 show a straight line relationship at lower light intensities and positive deviations from this straight line, L, at higher light intensities. This means that the quantum yields of fluorescence are constant only at lower light intensities and gradually increase at higher light intensities. The intensity at which such deviations become observable corresponds to the intensity at which photosynthesis begins to be light saturated. This increase in the fluorescence yield begins at lower light intensity in air than in nitrogen, and the increase is greater in air than in nitrogen at a given light intensity.

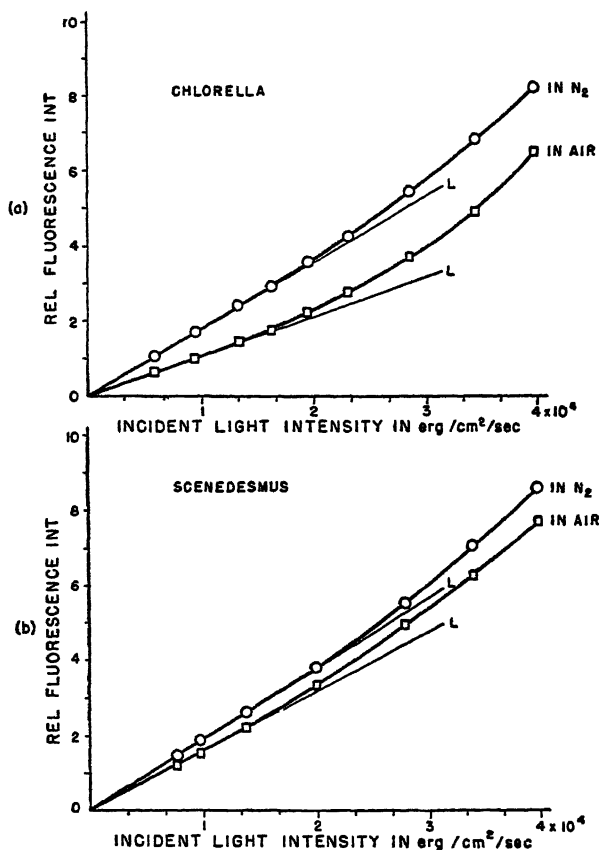


Fig. 6. Steady state fluorescence intensity vs. incident light intensity of (a) *Chlorella* and (b) *Scenedesmus* at 25°C., showing the increase in the fluorescence yield at high light intensities.

##### 5. Effects of Cyanide and Other Factors Limiting CO<sub>2</sub> Uptake

Fig. 7a shows the effects of  $10^{-3}$  M potassium cyanide upon the steady state fluorescence of *Chlorella* at room temperature at various light intensities. At this concentration, cyanide should cause almost total inhibition of photosynthesis. Curve (I) is measured with *Chlorella* in nitrogen in the presence of  $10^{-3}$  M potassium cyanide; curve (II), in air and in  $10^{-3}$  M cyanide; curve (III), in nitrogen without any cyanide; and curve (IV) in air without any cyanide. Comparisons of

the four curves show that both in nitrogen and in air the deviation of the curve from the straight line relationship becomes greater and begins at lower light intensities in the presence of cyanide than in its absence. Furthermore, this cyanide effect is more pronounced in air than in nitrogen.

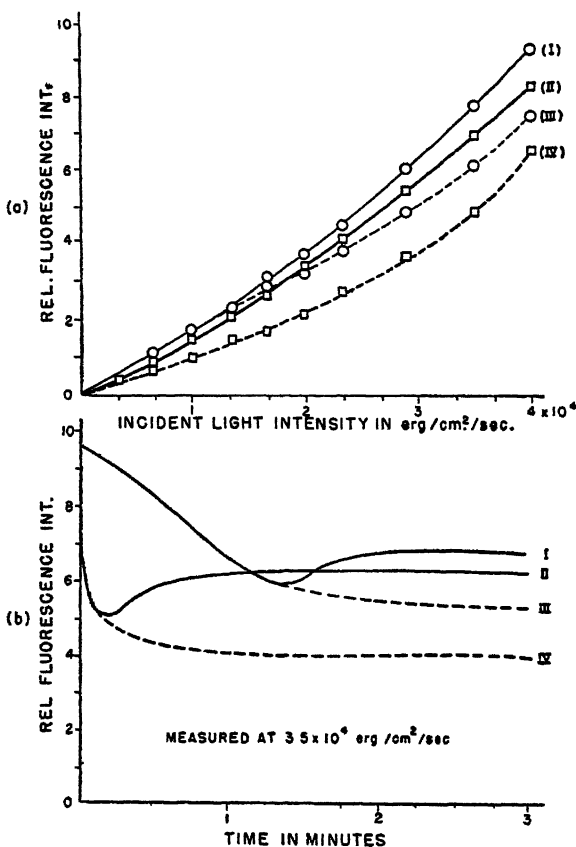


FIG. 7. Fluorescence behavior of *Chlorella* at 24°C., showing the effect of KCN in air and in nitrogen.

	Gas:	KCN:
(I)	N <sub>2</sub>	10 <sup>-3</sup> M KCN
(II)	Air	10 <sup>-3</sup> M KCN
(III)	N <sub>2</sub>	None
(IV)	Air	None

Fig. 7b shows the fluorescence time curves which were obtained with the same sample of *Chlorella* used in Fig. 10a at room temperature at the incident light intensity of  $3.5 \times 10^4$  erg/cm.<sup>2</sup>/sec. As mentioned above, curve (I) is in nitrogen in  $10^{-3}$  M potassium cyanide, (II) is in air in  $10^{-3}$  M potassium cyanide, (III) and (IV) are in the absence of cyanide in nitrogen and air, respectively. In both gases, the initial fluorescence changes are not affected by cyanide but the further fluores-

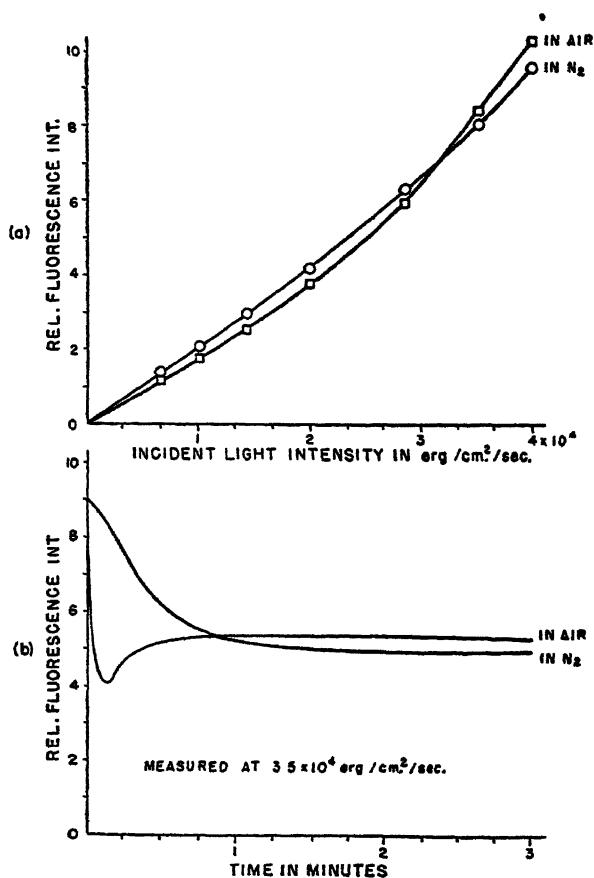


FIG. 8. Fluorescence behavior of a *Scenedesmus* culture at 24°C., showing the anomalous effect of air at high light intensities. This type of behavior was frequently observed in *Scenedesmus*.

cence decay is replaced by a rise after a short irradiation. This rise occurs earlier and is more pronounced in air than in nitrogen.

The same effects caused by cyanide, as described above, can also be brought about by low temperature and lack of  $\text{CO}_2$  supply, as our observations have shown, but we do not need to reproduce them by diagrams here. Also closely related to the influences of cyanide is that of unusually high light intensity on the fluorescence behavior of some old cultures of *Scenedesmus*. Fig. 8a shows the steady state fluorescence intensity *versus* light intensity curve at  $24^\circ\text{C}$ . of a culture of *Scenedesmus*. The deviation of the curve in air from the straight line relationship is much more pronounced here than in Fig. 6b. It is, in fact, so pronounced that the curve in air eventually crosses the curve in nitrogen at high light intensity. The fluorescence-time curve of the same sample of *Scenedesmus* at  $24^\circ\text{C}$ . is shown in Fig. 8b. The curve in air in this figure is similar to the curve in air in Fig. 7b, except that here the steady state fluorescence in air rises higher than that in nitrogen.

#### 6. Effects of Alkali, Quinone, and *o*-Phenanthroline after Anaerobic Incubation

The inhibition of photosynthesis in oxygen-free gas atmospheres after long anaerobic incubation was studied by Willstätter (36), Noack (27), and Gaffron (14), and recently by Franck *et al.* (11). Noack reported a slow removal of this inhibition by the addition of quinone or alkali, while Franck and co-workers found no such effect caused by the pH change.

The fluorescence behaviors of young and healthy cultures of algae were not influenced within half an hour by the presence of quinone and alkali, either in air or in nitrogen. However, after long anaerobic incubation of about 12 hours, the induction phenomenon disappeared and the steady state intensity decreased within one minute after making the solution  $10^{-3} M$  with respect to quinone at  $26^\circ\text{C}$ . and  $3.0 \times 10^4$  erg  $\text{cm}^2/\text{sec}$ . This is shown in Figs. 9a and b.

Whether, and to what extent, the fluorescence behavior of algae after anaerobic incubation is affected by alkalinity depends on the internal conditions of the cultures. Fig. 9c shows one example of the effect of alkalinity when the suspension was made alkaline to pH 9 after a long period of anaerobiosis.

The steady state intensity dropped to about 20% lower than its original value after a transient rise. The length of the period before the fluorescence drop occurs also varies with different samples of algae.



The effect of  $10^{-4}$  *M* *o*-phenanthroline in increasing the fluorescence intensity after anaerobic incubation before and after the addition of quinone is shown in Figs. 9a and b. The induction phenomenon was

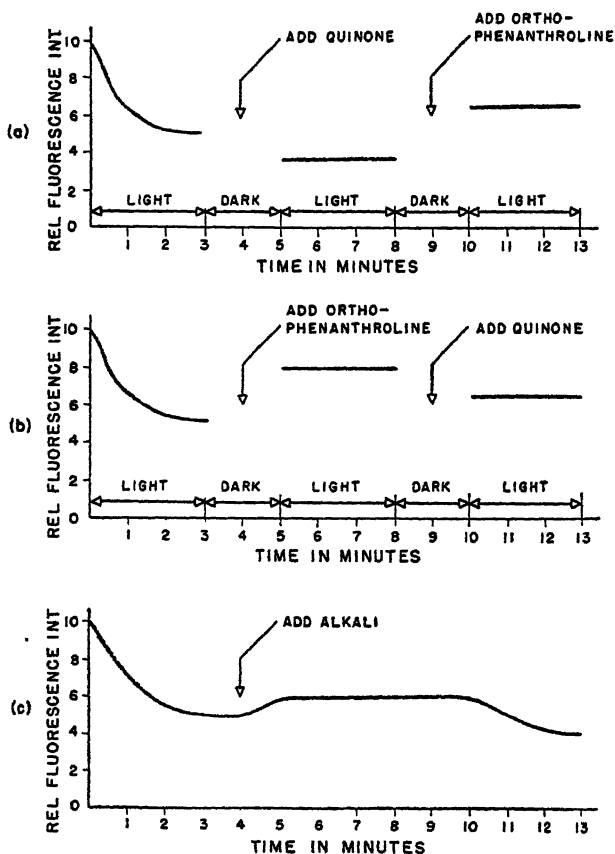


FIG. 9. Fluorescence time curves of *Chlorella* at 26°C. and  $3.0 \times 10^4$  erg/cm.<sup>2</sup>/sec., showing the effects of quinone, alkali, and *o*-phenanthroline.

also seen to disappear upon the addition of *o*-phenanthroline in the region of  $10^{-4}$ – $10^{-5}$  *M* used in the experiment.

The fluorescence intensity of algae was still affected by quinone when the latter was added 15 minutes after air had been admitted to the algae at the end of a long anaerobic incubation. This indicates that

the recovery of the algae cells to the conditions prior to the anaerobic incubation is a slow process. Old cultures of algae, after long storage in an icebox, sometimes show the same effect toward quinone as those young cultures after long anaerobiosis.

The method of staining the cells by methylene blue was used to test the permeability of the cell membranes. It was found under microscopic examination that the healthy algal cells before the anaerobic incubation are not stained by this dye. However, the cells immediately after the anaerobic incubation and the cells of old cultures, as mentioned above, were found to be stained by methylene blue.

## B. DISCUSSION OF ALGAE FLUORESCENCE

### 1. Fluorescence Time Courses during Induction Periods

*Fluorescence Time Curves in Air.* The fluorescence time curves represented in Figs. 3 and 4 measured in air for *Chlorella* and *Scenedesmus* need very little additional explanation beyond that given in the introduction. The quick rise of the fluorescence intensity at the beginning of the illumination is explained, like the corresponding one in leaves, as being caused by the narcotic surface layer, formed by the reaction between the photoperoxides and the carbohydrates.

The following decline of the curve corresponds to the gradual removal of the narcotic layer and poison through oxidation processes, giving the oxygen-liberating enzyme the opportunity to be gradually reactivated. After learning that the poisoning of the oxygen-liberating enzyme is caused by a metabolic product (11), it is possible to deduce more specifically the process by which the depoisoning of the enzyme takes place during illumination.

McAlister's and Franck, French and Puck's experiments have shown that, even after prolonged irradiation, each transition to a higher photosynthetic rate is connected with the development of induction phenomena. Consequently, during an illumination period the enzyme is not fully reactivated, but merely enough to keep up with the production of the peroxides and to remove the latter quickly enough to prevent noticeable oxidations of carbohydrates. In other words, the concentration of the poison in the dark is shifted to a lower level in the light by the illumination, and this level is the lower the higher the rate of photosynthesis. This reactivation is achieved by the reaction of the poison with a photosynthetic product. The end products of photo-

synthesis—carbohydrates and oxygen—cannot be the products responsible for this reaction, for they are either present in equal concentrations in the light and the dark, or they can be added in quantity during the dark period without removing the occurrence of the usual induction phenomena. It has to be a reaction with a short-lived photoproduct which causes the partial removal of the poison during irradiation. The only photoproducts which seem to be short-lived enough are the photoperoxides; if they remove poison by oxidation, the automatic adjustment of its concentration to the photosynthetic rate becomes understandable. The poison level will fall just so low that enough enzyme molecules are liberated to quickly remove the bulk of the peroxides made photosynthetically until the reaction rate between poison and the peroxides balances the slow rate of influx of poison to the photosynthetic apparatus.

*Secondary Induction.* No satisfactory explanation has been given hitherto for the frequently occurring second maximum of the fluorescence curves. Our interpretation is as follows: The concentration of the photosensitive reducible substance (namely, photosynthetic intermediates and the compound called RCOOH by Ruben *et al.* (31) which is formed by a dark carboxylation reaction preceding the photosynthetic steps) in the dark is higher than its steady concentration during illumination periods, if the  $\text{CO}_2$  uptake is the limiting factor, or one of the limiting factors, of the photosynthetic activity. Several processes, in fact, progress simultaneously at the beginning of an irradiation period: the decline of the level of the substance which poisons the oxygen-liberating enzyme, the production and removal of the narcotic, and the lowering of the concentration of the reducible substances.

The narcotic and the reducible substances compete for the places at the surface of the chlorophyll, according to the general rules of competitive adsorption. Not only the relative strength of the adsorption forces, but also the relative concentrations of the competing substances, are responsible for the distribution of chlorophyll surface between the narcotic and reducible substances. The concentration of the narcotic rises quickly to a maximum and falls from there on slowly during irradiation, together with the level of the poison for the oxygen-liberating enzyme. But the amount of chlorophyll surface occupied by the narcotic (and, consequently, the fluorescence yield) will depend on the concentration ratio of the narcotic and reducible substances.

If the concentration of reducible substances would remain constant and high, the decay of the fluorescence would follow the path of Curve a of Fig. 10; if the concentration has a considerably smaller but con-

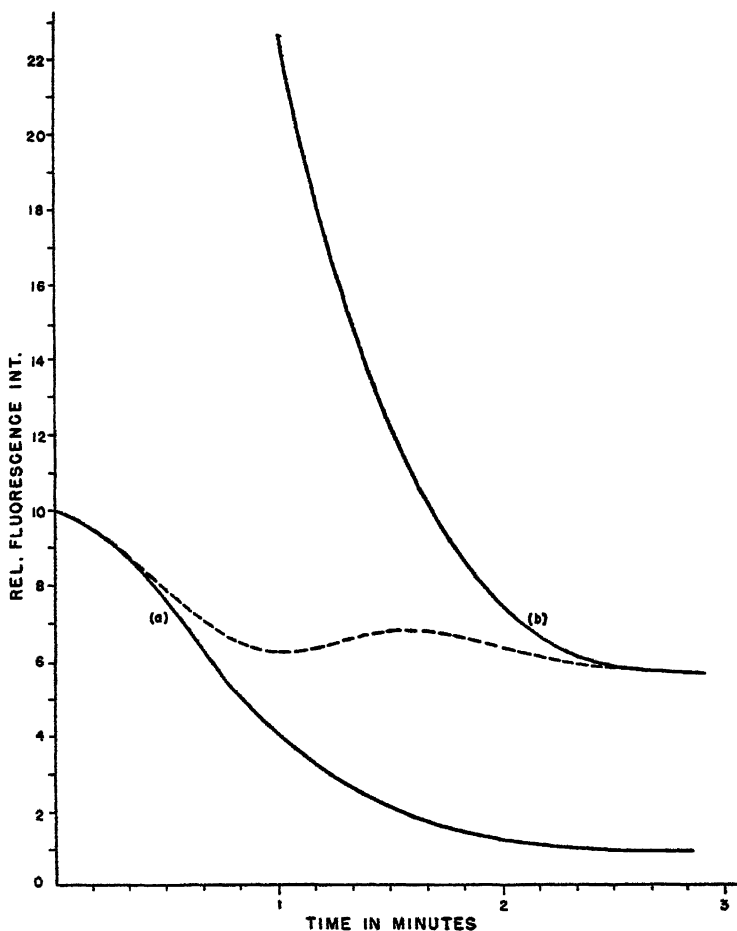


FIG. 10. Theoretical fluorescence-time curve at two concentrations of photosensitive, reducible substances with transition curve shown as dotted line.

stant value, Curve b would represent the time course. If, on the other hand, the reducible substances concentration is high, and stays high in the beginning, followed by an accelerated transition to the lower

concentration before all the narcotic has gone, the fluorescence intensity will follow first the path of Curve a and later that of Curve b with a transition between the two as indicated by the dotted line. The return of the fluorescence to a higher level during the transition indicates that the concentration of reducible substances falls temporarily more rapidly than that of the narcotic. Actually, the reducible substances concentration will stay constant at the beginning of the illumination until the chlorophyll surface is freed from the narcotic to such an extent that the photosynthetic consumption of the reducible substance surpasses its replacement by new carboxylation reactions. From that mo-

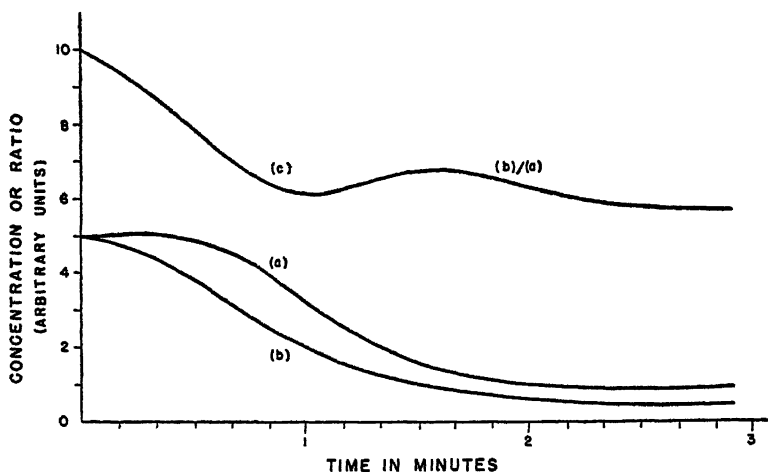


FIG. 11. Time course of concentration variation of narcotics (b), and photosynthetically reducible substances (a), and their ratio (b)/(a).

ment on, it will fall temporarily with a very steep slope while the photosynthetic rate may even surpass its steady state value. The concentration of  $\text{RCOOH}$  and the narcotic as a function of time may thus be represented by, respectively, curves a and b of Fig. 11. The ratio of the concentration of the narcotic to that of the reducible substance may then be represented by Curve c. This curve represents the amount of the narcotic adsorbed on the surface of chlorophyll, and therefore explains the fluorescence behavior of the secondary induction.

Our present observations on the relation between time of arrival of the second maximum and light intensity, as well as the observation

that all influences which lower the rate of the carboxylation reaction increase the height and duration of the second maximum (11) are in accordance with our interpretation.

*Effects of Cyanide.* Measurements of the influence of cyanide on the shape of the fluorescence time curves in air gave results which are in agreement with the measurements of Wassink *et al.* (34). Of theoretical interest is the fact that the first part of the curves are not influenced by cyanide even at a concentration of  $10^{-3} M$  which should reduce the steady state of photosynthesis to practically zero<sup>3</sup>. (See Fig. 7a.) This fact will be discussed later in connection with the fluorescence time curves in nitrogen.

Finally, an example of the abnormal curve shown in Fig. 8 may be mentioned. The curve in air is very similar to the curves observed in the presence of KCN. The resemblance of the fluorescence time curves taken with old algae to curves measured with KCN was observed by Wassink *et al.* This may be regarded as an indication that in such cultures the enzyme which is involved in the dark carboxylation reaction and responsible for the carbon dioxide uptake becomes the limiting factor just as in the cultures which are poisoned by cyanide. The same type of curve is also observed if the CO<sub>2</sub> uptake is limited by an insufficient supply of this gas in the algae suspension for normal photosynthesis.

*Influences of Cultural Conditions.* The great influence which the condition of algae cultures has on the fluorescence time curves strengthened very much our confidence in the hypothesis that the primary cause of the induction phenomena is a water-soluble excrete of the algae which poisons oxygen-liberating enzymes. The most important fact is the observation that some very young and dilute cultures show no induction phenomenon at all and the fluorescence intensity remains constant right from the start of the illumination. This indicates that the induction anomalies of the photosynthetic rates will also be absent. The concentration of the poison in the suspension will, of course, be small in young cultures, but also lack of permeability of the membranes of these young algae may play a role. This may be concluded from the observation that one of the young cultures did not show induction phenomena even after it was put for 24 hours in the culture solution

<sup>3</sup> Only the unusually high concentration of KCN used by Ruben *et al.* totally deprives the enzyme of any activity whatever and prevents even the very slow formation of RCOOH during dark periods.

which previously contained old cultures. The fact that not all young cultures of algae taken at the same age behave alike will mean that the permeability of each varies. Some show induction anomalies of the fluorescence, but they are systematically less pronounced than in old cultures. The other results on the influence of the previous history on the induction phenomena mentioned in the experimental part do not need further elaboration as they may all be explained on a similar basis.

*Fluorescence Time Curves in Nitrogen.* The fluorescence time curves measured in nitrogen are, as mentioned, of special interest for comparison with the time course of the rate of oxygen production measured under very similar conditions (11). Again there are two types of curves for the fluorescence intensity, as well as for the rates of oxygen production: the type shown in Fig. 3 with only one fluorescence maximum and the type shown in Fig. 5, in which a secondary maximum is superimposed on the fluorescence decay. Also, the anomalies caused by potassium cyanide are observed in the rate curves of oxygen production as well as in the fluorescence curves (see Fig. 7b); again the first part of the fluorescence curves is the same whether cyanide is present or absent just as the rate of oxygen production.

The effects of cyanide are supposed to inactivate the enzyme involved in the carboxylation reaction,  $\text{RH} + \text{CO}_2 \rightarrow \text{RCOOH}$ , according to Ruben *et al.* (31). If the enzyme is strongly, but not totally, poisoned, the velocity of the carboxylation reaction is greatly slowed down, but during a dark period of sufficient length, the reservoir of  $\text{RCOOH}$  molecules will be filled up to the same value which it reaches in the absence of potassium cyanide. As soon as the supply of  $\text{RCOOH}$  accumulated in the dark is used up upon illumination, a marked rise of fluorescence occurs, caused by the denudation of the chlorophyll of  $\text{RCOOH}$  and intermediates, which corresponds to the observed decay in oxygen production. A similar rise, but only after a shorter irradiation time, occurs in air, indicating that here the reservoir of  $\text{RCOOH}$  and intermediates is more quickly exhausted by photosynthesis than in pure nitrogen. This is to be expected as the higher initial values of the fluorescence intensity in nitrogen reveal that under anaerobic conditions another narcotic substance is made metabolically, probably by fermentation, and the rate of photosynthetic consumption of  $\text{RCOOH}$  is curtailed by the presence of this narcotic.

It must be emphasized that the rise of fluorescence, observed whenever the plant is deprived of reducible substances to be photosynthe-

sized, is predominantly caused by the deposition of a narcotic layer which covers a part of the chlorophyll. If the denudation takes place in air, the narcotic is made by photooxidation; if no oxygen is present, as in pure  $N_2$ , the narcotic substance made by fermentation settles down on the chlorophyll. It is not, therefore, justifiable to draw any conclusions from the fluorescence time curves measured in the presence of cyanide in regard to the problem of whether really denuded chlorophyll fluoresces more than the chlorophyll in contact with  $RCOOH$  and photosynthetic intermediate products.

The general shape of the fluorescence time curves in pure  $N_2$  in the presence of enough carbon dioxide can be understood only if it is taken into account that two natural narcotics are present under these conditions. One is slowly made by anaerobic metabolism and is shown by the high value of the fluorescence intensity at the start of the illumination after the algae remained for a long time in the dark anaerobically. The other is made during illumination by the reaction of the peroxides with carbohydrates. The first-mentioned has its maximum concentration at the start of the illumination, and then decreases under the influence of the light since this narcotic is more quickly removed by the attack of photoperoxides than produced by anaerobic metabolism. A dark period of one or two minutes, which suffices for the development of the induction phenomena in air, does not restore the high fluorescence intensity at the start of illumination in nitrogen. The conclusion that this narcotic can be removed by a reaction with photoperoxides was drawn from measurements of the light saturation curves of the oxygen production with algae under anaerobic conditions (11).

Curve a of Fig. 12 shows the theoretical fluorescence time curve if this metabolically produced narcotic were alone present. The second narcotic which is the usual one responsible for the induction phenomena in air, should have in nitrogen a time course of concentration variation as indicated by curve b. It produces a rise of fluorescence similar to, but smaller and somewhat slower than, that in air, since the photosynthetic peroxide production is depressed by the presence of the first narcotic. But the rise of the fluorescence intensity caused by the second narcotic in an oxygen-free atmosphere is not followed by decline, as the oxygen-liberating enzyme does not recover under these conditions and, correspondingly, the production rate of the narcotic remains constant and high. Addition of Curves a and b gives Curve c



which shows the combined influence of both narcotics on the fluorescence time curve in accordance with our observations.

The reason for the negligible recovery of the oxygen-liberating enzyme under anaerobic conditions, in spite of the constant removal of the poison by its reaction with peroxides, is given by two factors. The first one is the great reservoir of poison which is accumulated in the water throughout the anaerobic treatment, and the likelihood that the poison has easy access to the enzyme, since the cell membranes become permeable after anaerobic incubation. (See the experiments on membrane permeability in the following section.) The second reason is the

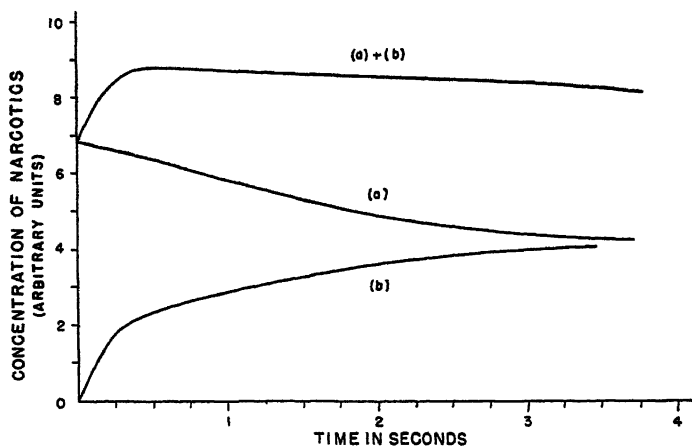


FIG. 12. Time course of concentration variation of two narcotics and their total concentration which influences fluorescence intensity of chlorophyll.

apparently higher production rate of the poison by anaerobic than by aerobic metabolism. Revealing in that respect is the slow decline of the fluorescence intensity to the same values as observed in air, if oxygen is allowed to accumulate in the cell suspensions.

*Effect of Alkali, Quinone and o-Phenanthroline after Anaerobic Incubation.* The effect of the addition of alkali on the fluorescence behavior under anaerobic conditions was originally investigated to clear up an apparent contradiction between the results of Noack and co-workers (27) and of Franck and co-workers (11). The former observed an effect of the pH change in removing anaerobic inhibition of photosynthesis, while the latter found none.

It was found that the cell membranes become more permeable after a long anaerobic incubation and that the fluorescence intensity of algae eventually decreases upon making the suspension alkaline. This is in agreement with our assumption that the narcotic substance responsible for the high fluorescence yield of chlorophyll is an acid and loses its surface-active properties when neutralized. The increase in permeability of the cell membranes caused by prolonged anaerobic incubation thus explains the difference between the observations of Franck *et al.* and Noack *et al.* The former group used only 2–3 hours of anaerobic incubation while the latter group used 15–24 hours.

Since the alkali effects in chloroplasts will be discussed later, it suffices to discuss only briefly one example of the alkali influence on algae fluorescence shown in curve c of Fig. 9. The algae used have been under anaerobic conditions for 15 hours. Alkali is added anaerobically when the steady state is reached 4 minutes after the start of the illumination. The pH of the solution, which has previously been 6.8, goes up to 9.0. The fluorescence intensity rises in about one minute, stays high for about 7 minutes and then gradually decreases to a steady state value lower than that before addition of alkali. The temporary rise is only a secondary phenomenon as a result of the CO<sub>2</sub> limitation caused by the sudden alkalinity of the solution. The important point is the removal by alkali of the narcotic layers present in algae under anaerobic conditions with the result that the steady state fluorescence is eventually lowered.

Also the permeability to other substances, like quinone and *o*-phenanthroline increases greatly after long anaerobiosis, as can be seen in Figs. 9a and b. If quinone is added in darkness, or during illumination after long anaerobiosis, no induction fluorescence anomalies occur upon renewed illumination, and the fluorescence is weaker than the steady state values before the addition of quinone. Quinone removes the narcotic layer on chlorophyll either by oxidizing the narcotic away or by displacing it. Quinone is photosensitive, as Warburg (35) showed that it is reduced to hydroquinone in the presence of chloroplasts while oxygen is evolved. Experiments are planned for studying the oxygen production with reduction of quinone in whole algae in the future.

*o*-Phenanthroline seems to have the properties of a narcotic. In its presence the fluorescence is, therefore, high and constant throughout an illumination, though not as high as in the presence of other narcotics like urethane.

## 2. Steady State Fluorescence

Wassink and Katz found that with *Chlorella* at room temperature and in the presence of enough  $\text{CO}_2$  the steady state fluorescence intensity rises linearly far into the light saturation region of photosynthesis. Our curves for *Chlorella* and *Scenedesmus* show, under similar conditions, a definite deviation from the straight line relation, indicating that the fluorescence yield rises in the region of higher light intensities. Seen from the point of view of the kinetic theory of photosynthesis, this difference, if real, indicates that in Wassink and Katz's case the limitation responsible for saturation was very predominantly caused by the maximum capacity of the enzyme which stabilizes the freshly formed photoproducts (9), while, in the present observations, the limitations of the rates of formation of  $\text{RCOOH}$  share partly the responsibility for the occurrence of light saturation. If the latter is the case, a part of the chlorophyll is denuded of  $\text{RCOOH}$ , etc., with the effect, discussed previously, that a narcotic is deposited in air as well as in pure  $\text{N}_2$ . The fact mentioned in the introduction that the rise of the fluorescence yield is small in nitrogen containing enough oxygen to prevent fermentation formation of a narcotic and, on the other hand, so little oxygen that the production of a narcotic by photooxidation is small, is in perfect agreement with the views expressed. Deficiency of  $\text{RCOOH}$  alone (if the narcotic is removed by alkalis) would change the fluorescence yield very little, if at all. All influences which reduce the efficiency of the carboxylation reaction, such as low temperature, lack of  $\text{CO}_2$  or addition of  $\text{KCN}$ , cause denudation and, correspondingly, a higher rise in the fluorescence yield, and this rise begins at lower light intensities than in the case of the uninhibited  $\text{RCOOH}$  formation.

## C. FLUORESCENCE OF LEAVES

The following exploratory experiments on the fluorescence of leaves in air were made with the intention of testing whether certain inhibitions of photosynthesis are connected with changes of the fluorescence intensity.

### 1. Excised Leaves

Harder (16) observed that higher plants, growing in strong light, often reduce their photosynthetic activity after a few hours of strong illumination and resume the original rate of photosynthesis only after

several hours of rest. This occurs under constant external conditions. Kursanow (21) has shown that the degree of this temporary inhibition is increased by the presence of an ample food supply in the plants. The question arises whether such adaptation of the photosynthetic rate to the food supply available in the plant is caused by the production and removal of narcotic surface layers. To test this idea, the following experiments on leaves were made.

The fluorescence intensity of a leaf, which is attached to the plant so that the carbohydrate may flow into the plant, was compared with the fluorescence intensity of the same leaf cut off from the plants. Fig. 13b shows the fluorescence time curve at 25°C. and  $3.0 \times 10^4$  erg/cm<sup>2</sup>/sec. of a tobacco leaf *in situ*, and Fig. 13a shows that of the same leaf cut off the plant at the distal end of the petiole, each curve being meas-

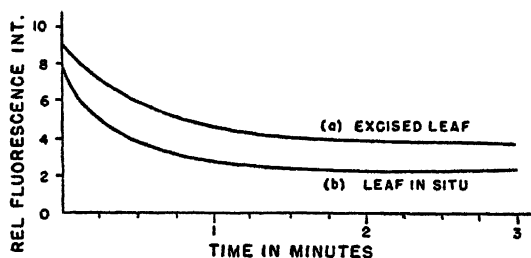


FIG. 13. Fluorescence time curves of a tobacco leaf, showing the effect of excising it from the plant.

ured after a dark period of 5 minutes. On comparing the two curves, it is seen that the induction maximum is higher and is followed by a slower fluorescence decay to a greater steady state intensity in the excised leaf. It required about 30 minutes of strong illumination for these differences to develop in the excised leaf, but once fully developed, there was little change for 2 or 3 hours more of illumination.

No direct comparison was made between changes of the fluorescence and changes of the photosynthetic output by the same leaf. For the experiments on changes of photosynthetic activities of excised leaves, we relied upon the numerous observations made by Willstätter and Stoll (36), who found that the photosynthetic activity of an excised leaf declined with the duration of irradiation. Furthermore, measurements were made by Aronoff (1) who showed that tobacco leaves from the same plant material as ours show, for the first 45 minutes, a quick decay of their photosynthetic rate, followed by a much slower decay. The change of the fluorescence intensity again seems to be antiparallel to that of the photosynthetic rate.

If an excised leaf had been left in the dark for about 15 hours with its stem immersed in a nutrient solution and was then taken out and illuminated with white light of about 40,000 lux, we found the following effect. After the first few minutes of illumination, the fluorescence time curve may also be represented by Curve b of Fig. 13. After about 1-2 hours of continuous illumination, the fluorescence time curve of this excised leaf had changed to one that may be represented by Curve a of Fig. 13. The induction loss has thus increased and the steady state fluorescence intensity is now about 30% higher. Further illumination for more than 3 hours does not cause any more change in its fluorescence behavior.

With the leaf *in situ*, however, no such effect of illumination is observed. The fluorescence time curve of such a leaf remains the same throughout 3 hours of illumination after it has been kept in the dark for 15 hours. Now if this leaf is cut off from the plant, the fluorescence behavior of the leaf is again as that described above for the excised leaf.

The same kind of increase in fluorescence intensity from curve a to curve b for an excised leaf caused by illumination, as described above, may often be brought about also if the leaf is immersed in a 10% sucrose solution. The leaf was left in the dark overnight as before and the fluorescence curve measured. Now its stem is immersed in a 10% sucrose solution and left in the dark for about 2 hours. The fluorescence time curve measured right after this treatment shows a prolongation of the induction period and an increase in steady state intensity of about 30%.

The overall result gives support to the assumption that the presence of ample food raises the level of the substance which inactivates the oxygen-liberating enzyme during the dark as well as the illumination periods. That is in line with our general picture. If the poison is a product of metabolism, its production rate is supposed to grow with the amount of substrate to be metabolized. (It is also possible that surplus of food may favor especially the production of the poisoning metabolic product.) A mechanism presents itself here by which higher plants can protect themselves against overproduction of photosynthetic substances. Whenever under prevailing external conditions—long hours of sunshine in the presence of  $\text{CO}_2$ —an overproduction threatens, the oxygen-liberating enzymes which convert peroxide into oxygen become

partially poisoned. This causes the formation of a narcotic layer, which, in turn, reduces the photosynthetic activity to a level adequate for the conditions of the plants. It becomes possible to explain by this process the shapes of the rate-time curves of photosynthesis in Harder's "sun plants" and "shadow plants," but the experimental evidence of the connection between the periods of photosynthetic inhibition and the formation of a narcotic layer has to be broadened before a detailed discussion is justified.

## *2. Pressed Leaves*

It is known that any damage done to the plant cells reduces considerably, or even stops, photosynthesis. Willstätter and Stoll showed that even pressing a leaf suffices to diminish its activity to very small values. Aronoff, who repeated these experiments, found that the damage done by pressing can be removed if sufficient time is given to the leaf to recover. Experiments have been undertaken to find out what kind of changes in the fluorescence yield are connected with the depression of the photosynthetic rate caused by the pressing of the leaf and with the recovery process.

Fig. 14a shows the fluorescence time curves of a leaf before being pressed and after being pressed, respectively. Fig. 14b shows the corresponding initial changes in the first 3 sec. on an expanded scale. A slower initial rise to the maximum followed by a slower decay to the steady state was observed after the leaf was pressed. These effects increase with the degree of pressing.

An accurate and quantitative comparison of the fluorescence intensities of the leaf before and after pressing is not justified, as the optical properties of the leaf surface may have been changed by such pressing. However, the differences in the slopes of the initial changes are not affected by such change in optical properties. The fluorescence time curve after recovery is the same as the curve before pressing.

The experiments show that the changes in the fluorescence behavior produced by pressing are just as reversible as the changes in the photosynthetic rates, but it is premature as yet to state the cause of such an inhibition.

## D. FLUORESCENCE OF CHLOROPLASTS

The chloroplasts are supposed to contain the whole photosynthetic apparatus and yet, when isolated from the cell, they show only a weak

and shortlived photosynthetic activity. The activity became qualitatively observable when Molisch (23) and Beijerinck (3) used luminous bacteria as an indicator for the evolution of tiny amounts of oxygen by photosynthesis. Recently, Franck (6) was able to make quantitative measurements of the time course of the rate of photosynthetic oxygen production by using the newly developed micromethod (28).

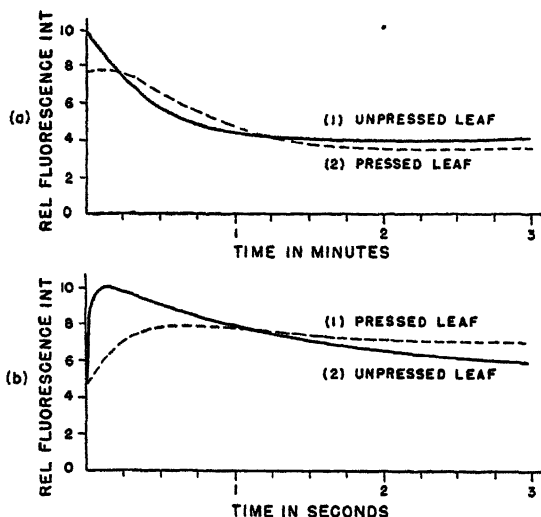


FIG. 14. Fluorescence time curves of an excised tobacco leaf, showing the effect of pressing it in (a) at low speed recording and (b) at high speed recording.

The great limitation of photosynthetic activity of the chloroplasts is not due to the inability of chlorophyll to induce photochemical reaction, as shown by the observation of Hill and co-workers (17) and of French and co-workers (12), who extended Hill's work. They found that isolated chloroplasts are able to cause reduction of ferric oxalate in an aqueous solution and to liberate considerable quantities of oxygen thereby. Finally Warburg observed that quinone was reduced to hydroquinone in the presence of chloroplasts in an aqueous solution, and again oxygen production (one oxygen molecule for every two reduced quinone molecules) is connected with that process.

On the basis of his measurements of the time course of oxygen production and other evidence, Franck came to the conclusion that the limitation of the photosynthetic activity in isolated chloroplasts is caused by the lack of  $\text{RCOOH}$  formed by the dark carboxylation reaction:  $\text{RH} + \text{CO}_2 \xrightarrow{\text{enzyme}} \text{RCOOH}$ . Either  $\text{RH}$  is present in too small a concentration in the chloroplasts or the enzyme involved is available only in very insufficient amounts in the plastids. The steady photosynthetic rate is then

limited by the low production rate of  $\text{RCOOH}$  while the rate at the beginning of the irradiation is greater because of the reserve of the intermediates and  $\text{RCOOH}$  complexes, which are either left over or slowly accumulated during the preceding period of darkness. Fluorescence studies may be used to test this hypothesis.

Measurements of the fluorescence of the grana of chloroplasts are reported only once in the literature, in the form of a letter to the editor from H. Kautsky and W. Zedlitz (20), which, however, contains important information in a condensed form.

### 1. Fluorescence Time Curves of Chloroplasts

No essential difference is observed between the fluorescence behavior of chloroplasts taken from tobacco and spinach leaves, although we find that the chloroplasts of spinach leaves are more sensitive to temperature, illumination and other factors. It is, therefore, more difficult to obtain reproducible results with spinach chloroplasts on the fluorescence behavior in different gases. The results reported below on the fluorescence time curves are typical of what are generally obtained from a number of observations using tobacco chloroplasts. The quantitative difference of fluorescence intensity in different gases varies appreciably, however, with the history and internal conditions of the chloroplasts.

Figs. 15a, b, and c show the fluorescence time curves of the intact chloroplasts in oxygen, in air, and in nitrogen, respectively, at  $4^{\circ}\text{C}$ .

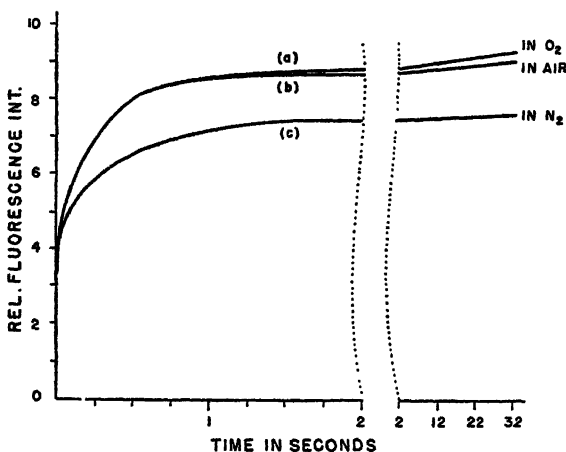


FIG. 15. Fluorescence time curves of intact chloroplasts at  $4^{\circ}\text{C}$ . and  $3.0 \times 10^4 \text{ erg/cm}^2/\text{sec}$ . in various gases.



and at the usual exciting light intensity of  $3.0 \times 10^4$  erg cm<sup>2</sup>. sec. The initial fluorescence rise of each curve resembles the initial fluorescence rise of whole leaves but is slower. Instead of being followed by the usual fluorescence decay, after the rapid initial rise in 2 sec., the fluorescence intensity increases slowly and uniformly, and becomes constant only after about one-half minute.

The initial values of fluorescence and the general shape of the curves were the same in air, in nitrogen, and in oxygen. The slope of the initial rise and the final height reached are, however, greater in air and in oxygen than in nitrogen. The phenomenon of initial fluorescence change recurs and is reproducible after a dark period of about 5 minutes in air and about 15 minutes in nitrogen, provided the chloroplasts are not damaged by the previous illumination.

Kautsky and Zedlitz using grana of *Saponaria* obtained the same curve in air as ours but observed a constant fluorescence intensity-time curve in nitrogen without any initial rise after the grana was once illuminated. The recovery period in their observations was 10-15 minutes in air but no recovery occurred in pure nitrogen. Addition of traces of potassium ferrioxalate or quinone to the grana suspension in nitrogen, however, gave the same type of curve as in air. They also mentioned results of measurements with grana of spinach. The recovery period in air is much faster with these grana. It takes only about one minute. No observations were reported about measurements with spinach grana in nitrogen.

Fig. 16 shows the fluorescence time curves of four samples of intact chloroplasts taken from the same preparation used for the measurements of Fig. 15. All these curves are measured in air at 4°C. and the usual light intensity of  $3.0 \times 10^4$  erg cm<sup>2</sup>. sec. one-half hour after isolation and under the same conditions except for the factors indicated below. Curve a was obtained in the presence of .02% phenyl urethane; curve c was obtained at a lower exciting light intensity of  $2.2 \times 10^4$  erg cm<sup>2</sup>. sec.; curve d was obtained after 10 hours of standing in air at 25°C.; and curve b was the normal curve obtained in air.

The fluorescence intensity in the presence of phenyl urethane was constant throughout an illumination and was close to the final fluorescence intensity in the absence of phenyl urethane. Kautsky and Zedlitz also observed such constancy in fluorescence intensity without an initial rise in the presence of phenyl urethane.

The extent of initial rise of the fluorescence intensity increased with light intensity at low intensities and became nearly constant at about  $2 \times 10^4$  erg/cm<sup>2</sup>. sec. The slope of this initial rise when plotted against light intensity also shows the same kind of light saturation effect. The saturation light intensity for the slope of the initial rise varied greatly with different samples of the chloroplasts. However, it was found that the saturation intensity is always considerably lower in chloroplasts than in leaves.

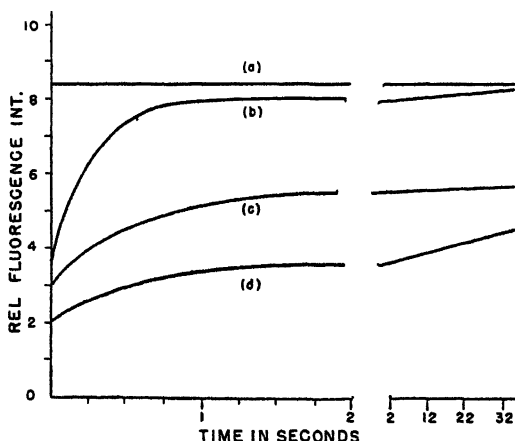


FIG. 16. Fluorescence time curves of intact chloroplasts in air, at 4°C. and  $3.0 \times 10^4$  erg/cm<sup>2</sup>/sec., and  $\frac{1}{2}$  hour after isolation, unless otherwise specified. (a) In 0.02% phenyl urethane, (b) normal curve, (c) at  $2.2 \times 10^4$  erg/cm<sup>2</sup>/sec., (d) 10 hours at 25°C. after isolation.

The increase in the age of the preparation (that is, the lapse of time after the preparation of the chloroplast suspension) decreased the slope and the extent of the initial rise. This effect was greatly accelerated by bubbling gas through the chloroplast suspension, and therefore, whenever possible, bubbling was avoided. Standing at room temperature for a few hours or being illuminated strongly for a few minutes decreased greatly the slope and magnitude of the initial rise. However, the history and internal condition of the chloroplasts seemed to play important roles in determining the sensitivity of the chloroplasts to these factors. Lack of carbon dioxide supply and the presence of hydrogen cyanide, however, had no influence on the initial fluorescence change.

Generally, the fluorescence behavior of the burst chloroplasts was

similar to that of the intact chloroplasts except for the fact that the burst chloroplasts showed a more pronounced effect of the age of the preparation and were more sensitive to higher temperature and to strong illumination.

## 2. Influences of Oxidizing Agents

Fig. 17a shows the effect of  $10^{-3} M$  quinone upon the fluorescence behavior of intact chloroplasts at  $4^{\circ}C.$  and  $3.0 \times 10^4$  erg/cm<sup>2</sup>/sec.

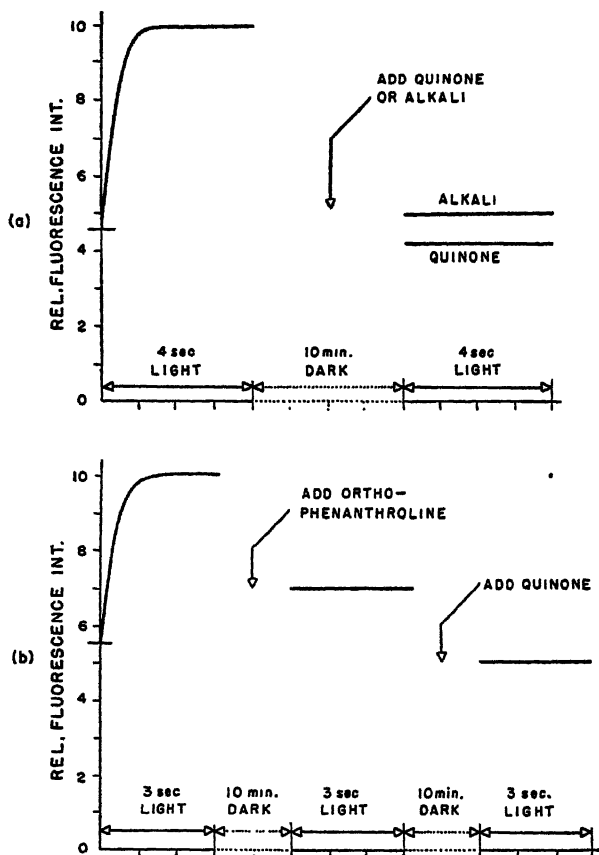


FIG. 17. Fluorescence time curves of intact chloroplasts at  $4^{\circ}C.$  and  $3.0 \times 10^4$  erg/cm<sup>2</sup>/sec., showing the effects of quinone, alkali, and *o*-phenanthroline.

The final fluorescence intensity was reduced to approximately 10% lower than the initial value at the start of illumination and remained constant at this value during the next illumination after a 10-minute dark period. The phenomenon of initial fluorescence change disappeared completely in the presence of a sufficiently high concentration of quinone, usually about  $10^{-3}$  *M*. Increasing the concentration beyond this value brought about little further change.

A freshly prepared  $10^{-3}$  *M* aqueous solution of purified quinone is slightly yellow in color, but it was found that its absorption of exciting and fluorescence light is negligible for the purpose of the fluorescence measurements. This was shown by the fact that quinone did not affect the fluorescence intensity of chloroplasts which had become inactive upon long standing in air and, therefore, showed no initial fluorescence change upon illumination. The possibility of chlorophyll bleaching by quinone within 10 minutes was eliminated by the fact that the concentration of chlorophyll measured colorimetrically, before and 10 minutes after the addition of quinone with illumination, showed no change. The quinone solution was neutral and did not influence the pH value of chloroplast suspensions.

The effects of quinone occurred in less than half a minute, which was the time usually required to thoroughly mix the quinone with the suspension in the apparatus used. The speed of this reaction was not affected by illumination or temperature, and was as rapid with intact chloroplasts as with burst ones. Washing of the chloroplasts several times did not restore the original fluorescence behavior.

### *3. Influence of Alkalies*

The pH of the chloroplast suspension in a 12% aqueous solution of sucrose was about 6 and was made more alkaline by buffering it with 0.1 *M* potassium phosphate to a pH of about 7.5. The effect of alkalies is similar to that of quinone in decreasing fluorescence intensity and in bringing about the disappearance of the initial fluorescence change.

Different from quinone, however, the effect of the alkalies on fluorescence intensity is less extensive. Alkalies decrease the fluorescence intensity to a value which is higher than, or the same as, the starting value but not any lower. The exact extent depends greatly on the internal conditions of the chloroplasts. Quinone decreases the fluorescence intensity further if it is added after the addition of alkalies,

but alkalis added after quinone will not decrease the fluorescence intensity any further.

Unlike that of quinone, the influence of alkalis is a slower process which has a high temperature coefficient and is accelerated by strong illumination. Phosphate buffer, for instance, had no effect upon the chloroplast suspension when it was kept in the icebox in darkness for as long as two hours at pH 7.5. Increase in the concentration of phosphate or in the pH value hastens this effect of decreasing the fluorescence intensity.

With different alkalis, the velocity of this reaction increased with the decreasing molecular or ionic size and with polarities of the alkaline substances. Ammonia, for instance, acts much faster than phosphate. With burst chloroplasts, however, the reaction, like that of quinone, is very rapid and practically independent of the various factors, including temperature, illumination, concentration, and pH value, as discussed above.

#### 4. Influences of *o*-phenanthroline

Inhibition of photosynthesis by *o*-phenanthroline was previously studied by Gaffron (14). Later, *o*-phenanthroline was reported by Warburg to inhibit the photochemical evolution of oxygen by illuminated chloroplast suspensions in the presence of quinone.

Fig. 17b shows the effect of  $10^{-4}$  *M* *o*-phenanthroline on chloroplast fluorescence before the addition of  $10^{-3}$  *M* quinone. Unlike its effect in algae, *o*-phenanthroline influenced the chloroplast fluorescence similarly to alkalis. It also caused a decrease in the fluorescence intensity and the disappearance of the initial change, but it decreased the fluorescence intensity by only 25%. Raising the concentration of *o*-phenanthroline to  $10^{-3}$  *M* brought about no further change. Addition of quinone to the chloroplast suspension after the addition of *o*-phenanthroline caused the fluorescence intensity to drop by about 25% more. However, the addition of *o*-phenanthroline after the addition of quinone to a fresh suspension had no effect.

#### 5. Steady State Fluorescence

Fig. 18 presents measurements of the fluorescence intensity of the steady state *versus* intensity of the exciting light at 4°C. The measurements with chloroplasts are not as reproducible as those with algae,

since illuminations long enough to attain the steady state fluorescence intensity will usually damage the chloroplasts by photooxidation. It can be seen, however, that the curves in air and nitrogen start to diverge from each other at a rather low light intensity and that the air curve rises considerably faster than the curve in nitrogen.

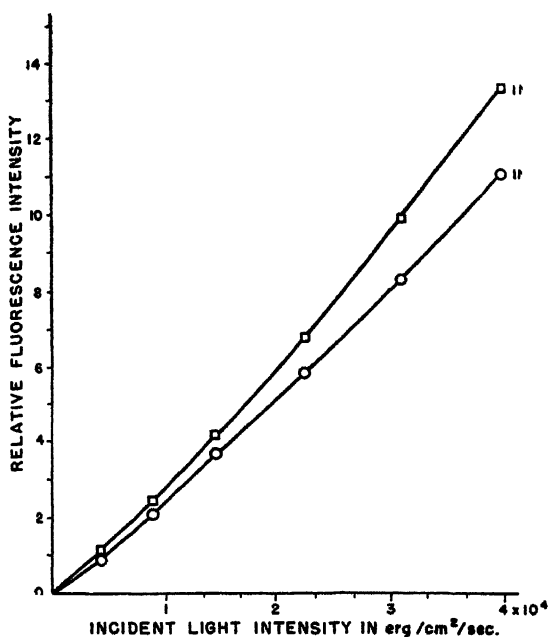


FIG. 18. Fluorescence intensity of intact chloroplasts (after one minute of illumination) vs. incident light intensity at 4°C. in air and in N<sub>2</sub>.

### 6. Discussion of Results

The initial rise of the fluorescence time curve of the chloroplasts resembles the one observed in leaves in shape and in relation to incident light intensity. We believe, therefore, that the same process is probably responsible for the initial rise in both cases. Peroxides produced in light and not removed quickly enough by the enzyme oxidizes carbohydrates to produce narcotic which enhances the fluorescence yield. In air the rise is quicker than in nitrogen as additional photo-

oxidation involving molecular oxygen can contribute to the production of the narcotic.

However, the slope of this rise is slower and the extent is smaller in chloroplasts than in leaves. It is as yet impossible to know all the causes of such differences. Apparently, the removal from chlorophyll of RCOOH and intermediates is one important cause. In addition, the decrease in concentration of the enzymes in the process of isolation and aging of the chloroplasts may be responsible.

At any rate, the initial rise is an indication that, at the start of illumination, photosensitive intermediates and maybe RCOOH are present, in agreement with the conclusion drawn from the measurements on oxygen production by chloroplasts (6). The fact that the initial fluorescence intensity is the same in air and in nitrogen, however, rules out the assumption that, in chloroplasts in nitrogen, a narcotic plant acid is made by anaerobic fermentation. The metabolism of the chloroplasts seems to be quite different, at least quantitatively, from that of whole cells. This is also shown by the fact that the narcotic made during illumination with peroxides is removed in air in the dark about 100 times slower in chloroplasts than in leaves. Respiration, which is supposed to be the main reason for the aerobic removal of the narcotic in leaves, must, therefore, be much less efficient in chloroplasts or may even be replaced by another oxidizing process. The slow removal rate of the narcotic must be the main cause for the fact that no fluorescence decay follows the initial rise as in leaves.

The removal of the narcotic during illumination certainly cannot be quicker than in the dark, and this slow removal is over-compensated by a slow formation of the narcotic by photooxidation as a result of the depletion of the photosynthetic apparatus, as previously discussed. The fluorescence curve, therefore, rises slowly after the rapid initial rise is over. This slower and later rise is more pronounced in oxygen and in air than in nitrogen since the denuded chlorophyll can sensitize photooxidation by molecular oxygen to produce more narcotic. As a result of this photooxidation, the fluorescence intensity, after about one-half minute of illumination, always lies higher than in nitrogen even at low intensities. This means that many chlorophyll molecules are deprived of RCOOH and intermediates even at such light intensities.

Kautsky and Zedlitz did not observe the recovery of the initial fluorescence rise after a dark period with *Saponaria grana*, whereas we

did with tobacco chloroplasts. However, as they found, recovery time seems, in general, to be very different in chloroplasts isolated from different species of plants. This may be caused by variations in the amount of oxidizing agents present in the chloroplasts of different origins. It is known that ferric ions are present in chloroplasts and this may be responsible for the removal of the narcotic during the dark period. In *Saponaria* chloroplasts and grana the concentration of such oxidizing agents may be insufficient for this removal process.

The influences of quinone, alkali, and *o*-phenanthroline on chloroplasts are in accordance with the results gained with algae after long anaerobic incubation. As mentioned before, these influences depend upon temperature, time of reaction, illumination, concentration, polarity, molecular or ionic size of the reactants, and whether the chloroplasts are intact or burst. All this indicates that a process of membrane permeation is involved as with algae. The fact that these substances act more rapidly in the chloroplasts than in algae results from the quicker permeation of the chloroplasts' membrane than of the algae cell membranes.

Again *o*-phenanthroline seems to have the properties of a narcotic and enhances the initial fluorescence intensity to a value which remains constant throughout illumination. Phenyl urethane is apparently more strongly adsorbed to the chlorophyll, as indicated by the higher intensity of the fluorescence.

The question of whether the experiments with chloroplasts can be used to show that denuded chlorophyll has a higher fluorescence yield than the chlorophyll engaged in the reduction of quinone deserves special mention. The difficulty that the denuded chlorophyll promotes photooxidation, which in turn causes the production of a substance with narcotic properties, can be avoided if this substance is prevented from settling down on the surface of the chlorophyll by the use of alkali. In principle, a comparison of the fluorescence intensity of chloroplasts suspended in an alkaline solution with the fluorescence intensity of the same suspension after addition of quinone should give a clear answer. The experiments showed that the addition of quinone to the alkaline solution generally lowered the fluorescence intensity, while addition of alkali to a suspension containing quinone has no effect. These results indicate that chlorophyll in contact with the reducible quinone has a smaller fluorescence yield than the denuded chlorophyll.



Finally, we may ask whether the fluorescence observations support Franck's hypothesis that the strong limitations of the photosynthetic activity in isolated chloroplasts is preponderantly caused by the lack of RCOOH formation. The enhancement of the fluorescence yield caused by the presence of oxygen, as well as the non-linear rise of the steady state fluorescence intensity with the intensity of the exciting light in air and in nitrogen, are indeed in accordance with the assumption that the chlorophyll is partly denuded and becomes more denuded by irradiation even with weak light (compare Fig. 18).

### SUMMARY

This paper includes a brief resumé of the facts and interpretations of the relationship between the fluorescence of chlorophyll and the photosynthetic activity in green plants. It then reports new observations on the fluorescence behavior of the following plant material:

- (1) Algae, under aerobic and anaerobic conditions, including induction phenomenon, steady state fluorescence, and influences of cyanide, quinone, alkalies, and other substances;
- (2) Leaves, under special conditions, including the effects of excising and pressing; and
- (3) Isolated chloroplasts, in air and in nitrogen, including the initial fluorescence rise, the final fluorescence intensity, and the effects of alkalies, quinone, phenyl urethane, and *o*-phenanthroline.

All observations, old as well as new, presented in this paper are in best accordance with the assumption (first used by Franck, French and Puck for an interpretation of the induction phenomenon of photosynthesis) that plants are able to produce natural narcotics. When the narcotics are present, they cover the surface of the chlorophyll, raise fluorescence yield, and suppress its photochemical activity. The narcotics seem to be some plant acids, as indicated by the influence of alkalinity. They are made, in some cases, metabolically; in others, by a photochemical oxidation of easily oxidizable substances like carbohydrates. The important physiological function of the narcotics is to control the photochemical activity of the plants. Harmful photooxidation and also overproduction of photosynthetic products can be prevented by this means.

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# An Osmotic Diffusion Pump

James Franck and Joseph E. Mayer

*From the Department of Chemistry, University of Chicago*

Received April 9, 1947

## INTRODUCTION

There exist, in living organisms, a considerable number of instances in which some material is transferred from a solution in which its concentration is low to a solution in which its concentration is higher. Included in this category are the cases in which the solvent, actually water, is transferred from a solution of relatively high solute concentration to one of low solute concentration, or where the transfer is from low hydrostatic pressure to higher, without compensatory changes in the solute concentrations.

Examples of solute transfer "uphill" occur in the kidneys, and in the intestinal walls of animals. Various somewhat surprising examples exist in which a solute, often an ionic salt, is at entirely different concentrations on two sides of a cell membrane, and although no net transfer is present, there does exist evidence that the wall is permeable to the solute. Although such effects as the Donnan equilibrium can occasionally account for this, there are also many cases in which evidence can be presented for the existence of a continuous supply of energy to maintain the gradient.<sup>1</sup>

As cases of the transfer of solvent "uphill" one may quote the flow of water from high solute concentrations to low solute concentration through the salivary glands, the passage of water through a frog's

<sup>1</sup> The literature on the subject is extensive. We may refer to the books of Hober, "Physical Chemistry of Cells and Tissues" (Blakiston, Philadelphia, 1946), and of Hoagland, D. R., "Lectures on the Inorganic Nutrition of Plants" (Chronica Botanica Co., Waltham, Mass., 1944). As an example of more recent literature, we may quote VISSCHER, M. B., FETCHER, E. S., JR., CARR, C. W., GREGOR, H. P., BUSHEY, M. S., AND BARKER, D. E., *Am. J. Physiol.*, **144**, 457, 468 (1945); **141**, 488 (1944); **142**, 550 (1944); *J. Phys. Chem.* **42**, 141 (1938); and *Proc. Soc. Exptl. Biol. Med.* **60**, 1 (1945).

skin, and the flow of fluids in trees. For the latter example other theories have been proposed which do not involve such a process, but it is suggested that the mechanism proposed here should be considered as a possible explanation for the root pressure.

In cases such as these the net process may be summarized by stating that material is transferred from low chemical potential to higher chemical potential. There is no overall conflict with thermodynamics, since there are many metabolic processes occurring in the same tissue that release free energy, but there does seem to be some mystery as to exactly what sort of mechanism can permit a chemical reaction releasing free energy to perform such a task as to pump water uphill, or to transfer a salt from a low to a higher concentration.

In this paper we wish to suggest a simple mechanism by which such things can be accomplished. An osmotic diffusion pump will be described, and its thermodynamic efficiency computed.<sup>2</sup>

#### QUALITATIVE DESCRIPTION

The essential unit of such a pump consists of the space between two membranes, in which a coupled chemical reaction, utilizing free energy supplied from outside, permits this unit to pump either solvent or dissolved solute into itself through one membrane and out through the other membrane, at a higher chemical potential than that from which it entered.

The schematic unit may be visualized as consisting of a tube containing some solvent, presumably water, and perhaps with solutes dissolved in it. At  $x = 0$ , and at  $x = l$  along the tube are two membranes. The osmotic diffusion cell is contained between these membranes. In the biological counterpart the "osmotic diffusion cell" may be part of the membrane wall of a living cell, or it may possibly actually be a living cell, or a series of cells, constituting part of the membranous tissue between two parts of the macroscopic organism. Such questions are immaterial to the description of the unit.

In the "osmotic diffusion cell" between the two membranes we suppose that there are various solutes to some of which the membranes are

<sup>2</sup> The authors do not possess enough familiarity with the vast literature to know whether and where similar ideas may have been suggested. Inquiries made of colleagues working in that field indicate that at least parts of our scheme have already been used to explain specific cases in a qualitative way. See, for instance, reference 3.

impermeable, preventing their diffusion out into the solution on both sides. The simplest case, that is clearly intended only to be schematic, is represented in the figure. For this case we assume that there are only two solutes,  $a$  and  $b$ , in this cell, and that  $b$  is an  $n$ -fold polymer of  $a$ . If  $D_a$  is the diffusion constant of  $a$ , and  $D_b$  that of  $b$ , we assume that  $D_a$  is less than  $n$ -fold greater than  $D_b$ , which means qualitatively that we assume that the same amount of material can diffuse more readily in the  $b$  form than in the dissociated form  $a$ , that is, that  $n$  molecules of  $a$  exert more drag on the solvent than one molecule of  $b$  containing the same atoms as  $n$  molecules of  $a$ .

Now let the conversion of  $na$  to  $b$ , and *vice versa* be inhibited in the solution, but assume it is catalyzed to equilibrium at the wall at  $x = l$ . Further, suppose the supply of some other material at the membrane at  $x = 0$  dissociates  $b$  to  $na$ , in excess of the normal equilibrium, by some chemical change in the supplied material which releases free energy.

Now there will be a constant cycling of the solutes  $a$  and  $b$ . The dissociated form  $a$ , produced at the wall at  $x = 0$ , will exist there at high concentration. It will diffuse to the wall at  $x = l$ , where it is at lower concentration, and be converted here, catalytically, to the associated form  $b$ , which has then higher concentration at  $x = l$  than at  $x = 0$ . The associated molecule  $b$  will diffuse from  $x = l$  to the wall at  $x = 0$  where it is reconverted to the dissociated form  $a$ .

Due to our assumptions about the relative diffusion constants,  $D_a/nD_b = \alpha < 1$ , the gradient in concentration of  $b$  will be less than that of  $a$  so the total concentration will be less on the right, at  $x = l$ , than on the left at  $x = 0$ .

Now we may consider any of three cases.

(1) The membranes may be impermeable to  $a$  and  $b$ , but permeable to the solvent and any other solute molecules it may contain. In this case the solution to the left and that to the right will be in equilibrium if the hydrostatic pressures of the solutions are different, namely, that on the right will be higher by the difference in osmotic pressure due to the difference in total solute concentration of the solution within the cell at the two walls.

(2) The membranes may be permeable to the solvent, impermeable to  $a$  and  $b$  and to other solute molecules in the solutions to the right and left. In this case we may have equilibrium with equal hydrostatic

pressures but with different solute concentrations on the two sides, that on the right being more dilute by an osmotic pressure difference equal to the difference between the osmotic pressures of the solution within the cell at the two walls.

(3) The third possibility is that the membrane is permeable to  $a$  but not to  $b$ . In this case we could have equilibrium between two solutions of hydrostatic pressures differing by the osmotic pressure difference due to  $b$  only at the two walls, but also of differing concentration of  $a$ , that on the left being the more concentrated.

Now in each case we have dynamic equilibrium between some material, or materials, existing at different chemical potentials on the two sides. In the first instance the solvent or solution has higher free energy on the right due to its higher hydrostatic pressure there. In the second case the chemical potential of the solvent would be higher on the right due to the lower concentration of solute. In the third case the solute  $a$  would have higher chemical potential in the left hand portion due to its higher concentration.

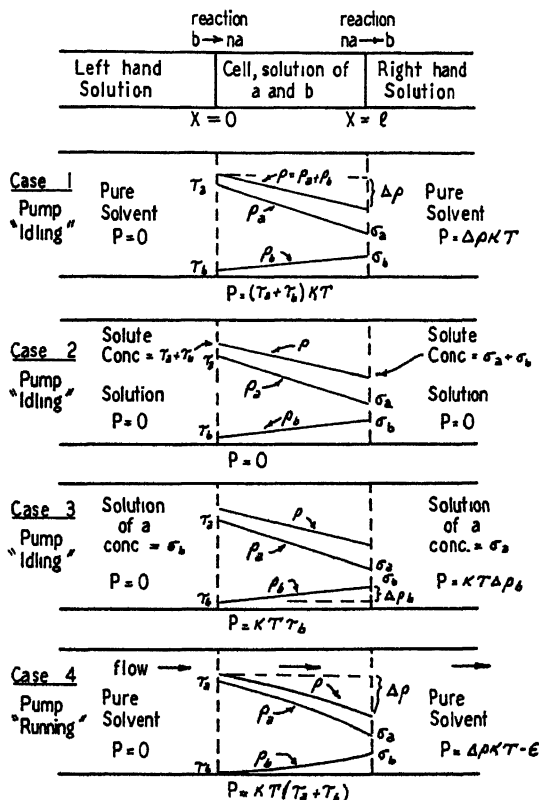
In each case we supply free energy to the cell to hold this dynamic equilibrium, that is, to keep the pump idling. Since no useful work is done the efficiency is zero, but the waste need not be large if the diffusion coefficients  $D_a$  and  $D_b$  are both small.

This equilibrium is dynamic, not static, by which we mean that a slight change in conditions can cause a flow to take place. If, in any of the examples, the rate of the reaction supplying free energy were to increase, the concentration gradients within the cell would increase. If, in the first case, the pressures were held constant, the solution would flow from the lower to the higher pressure. In the second example the solvent would be transported from the solution of high solute concentration to that of low. In the third case if the volumes on the two sides were held constant so that the small change in hydrostatic pressure could compensate for the change in the gradient of  $b$  the solute  $a$  would start to diffuse from the lower to the higher concentration.

In each case the flow would affect the concentration gradients within the cell in such a way as to counteract the flow, so that the rate of flow would be controlled by the rate of supply of energy to the chemical reaction.

Now, as previously pointed out, the efficiency of the pump is zero when there is no flow, simply because no useful work is being done.

As the rate of supply of free energy increases above that necessary to maintain the idling balance, a flow starts, and useful work is accomplished. The efficiency increases above zero. At first the flow is linear in the increase of energy supplied, but increases less rapidly as the increase is greater, and as the disturbance of the concentration gradients becomes too great. The ratio of expended free energy to that



gained, therefore decreases from infinity, at zero flow, to some minimum value, and then starts to increase as too rapid flow rates are maintained.

The mechanism of the device has been described in terms of the equilibrium established at the two membrane walls, which equilibrium is different for the two walls due to the concentration gradient established between them. Unless the coefficient of resistance to flow, pro-



portional to  $1/D_a$ , for the solute in the form of dissociated molecules  $a$  is greater than it is for the same amount of material in the associated form  $b$ , namely  $1/nD_b$ , the total concentration gradient in molecules per unit volume would not be, as we have assumed, such that the concentrations on the right are lower. Now we could have described the process in another manner. One could say that the molecules  $a$ , moving to the right, drag solvent with them, whereas those of  $b$ , moving to the left, counteract this. If, however, the frictional coefficient  $1/D_a$  for the molecules  $a$  is greater than that for the same amount of material, namely  $1/n$ th of a molecule of  $b$  moving in the reverse direction,  $1/nD_b$ , then the net drag is to the right. This force is adequate to move the solution or solvent to the right, even against a hydrostatic pressure head or an osmotic pressure gradient.

The picture in the third case of the solute diffusion is only slightly different. The molecules  $a$  have a tendency to diffuse to the right to the lower concentrations. This process is reversed by converting them to  $b$  at the right hand membrane surface, a membrane through which they cannot pass. They then diffuse in the opposite direction, in the form  $b$ , to the left hand wall where they are reconverted to  $a$ . By increasing the rate of reversion at  $x = 0$  one increases this transfer to the left, thus creating a net transport in this direction. The effect, and the direction of the effect, is independent of the diffusion coefficient values. However the efficiency does depend upon them. The net transport to the left in the form  $b$  dissipates less energy if the resistance term  $1/nD_b$  is small. Furthermore, if the concentration of  $b$  is high, the rate of flow for the individual  $b$  molecules is proportionally lower for the same transport, and since the energy dissipation per molecule is proportional to the velocity squared, there is less loss. The process becomes more efficient if  $D_a/nD_b$  is small and the concentration of  $b$  is high.

These qualitative considerations are adequate to establish the possibility of such mechanisms as those described. It fails, however, in answering an important question, namely: can such effects lead to processes whose overall efficiency is sufficiently high to make it appear reasonable that they may occur in nature? The answer to this question can only be given by setting up a schematic model and actually calculating the ratio of expended free energy to that gained as useful work. This ratio must exceed unity, but it would seem that if values as small as 10 or smaller are possible then it is not unreasonable to pre-

sume that nature might make use of the method. Actually, as we shall show, in the later sections of this article, values as low as three might be approached under favorable conditions.

The possible nature of the chemical reactions involved, and the problem of the specific transport of one ion, will be discussed in the following section.

### THE CHEMICAL REACTIONS

Before discussing the mathematical relationships necessary to compute the efficiency of such systems, we might briefly consider the possible nature of the chemical reactions involved. The schematic representation of a simple association-dissociation reaction, suggested in the previous sections, is hardly likely to be a mechanism used in nature. Presumably, if the type of mechanism suggested here is utilized in nature to convert chemical energy for transport purposes, it will be utilized with a wide variety of different chemical mechanisms, each suitable for each particular transport problem.

Whatever chemical reactions may be involved, it is necessary to supply some chemical species, at least to one of the membranes, in order to force the chemical reaction to proceed with an increase in free energy. It is not excluded that the reaction at *both* membranes is activated by another reaction, rather than merely catalyzed. In this case it is not even necessary, as we have assumed, that the solutes diffusing in the two opposite directions transport exactly the same numbers of the same kinds of atoms.

To be a little more specific about the possible nature of a reaction supplying energy, we might describe, in outline, one of the conceivable mechanisms. Suppose the solution bathing the one exterior wall of the cell, say that on the right, contains some solute of high reducing power, and the solution in contact with the other wall contains an oxidizing solute. If the solutes *a* and *b* within the cell are such that *a* is converted to *b* by oxidation, on the left, then energy is supplied by the reaction between the two compounds in the solutions on the two different sides of the cell. In such a case the chemical composition of *n* molecules of *a* would be different from that of *b*, but *b* might, for instance, contain more hydrogen atoms than *n* molecules of *b*, or *n* molecules of *a* contain more oxygen than one molecule of *b*.

It is quite immaterial, even, that we assumed *a* and *b* to be of vastly different molecular weights, that is, that *b* was described as a polymer

of  $a$ . It is important, if the *solvent* is to be transferred, that the diffusion constants of the two forms  $a$  and  $b$  should be considerably different for equal amounts of material. The mechanism would work as well if  $n$  were unity, and  $a$  and  $b$  were merely isomeric forms, provided only that the ratio  $\alpha = D_a/D_b$ , of the diffusion constants were less than unity.

In the case that solute is pumped from low to high concentration, it is essential that one of the solutes in the cell, namely  $a$ , actually be the solute which is concentrated in the net process. It is, of course, by no means necessary that the solutes in the cell be limited to two in number, and presumably, in any real case, there would be a considerable number of different solute materials present, diffusing in different directions. However, one of these must be the material transferred, since the membranes must be permeable to this solute, and one of the solutes, namely  $b$  in our simple example, must contain this material in some combined form such that the membrane is impermeable to this compound.<sup>3</sup>

When an ionic salt, say NaCl, is concentrated by such a cell, a possible form of the "associated" molecule  $b$  is suggested by the ion exchange resins. A soluble molecule,  $H_nR(OH)_n$ , might diffuse with the free ions,  $Na^+$  and  $Cl^-$ , to the right. The molecule must not react with the ions in solution, but when catalyzed by the membrane walls we might suppose the reaction:



to take place. The molecule  $Na_nRCl_n$  must then be soluble, and diffuse to the left, playing the role of our molecule  $b$ . At the left hand membrane the reaction above is to be reversed by the expenditure of free energy of some other reaction.

Even this hypothesis is almost obviously oversimplified. It is more reasonable to suppose that there are actually two such molecules,  $H_nR$ , and  $R^1(OH)_m$ , exchanging with the positive and negative ions

<sup>3</sup> Verzar, E., *Ergeb. Physiol. biol. Chem. expil. Pharmacol.* **32**, 391 (1931) and Wilbrandt and Laszt, *Biochem. Z.*, **259**, 398 (1933) explain the quicker resorption of hexose than that of pentose in the gut as due to phosphorylation of the former in the gut epithelium which increases the concentration gradient and thus speeds the diffusion in spite of its being the larger molecule. This phosphorylation may well be a specific reaction which occurs in the type of concentration cell we propose. The concentration problem is not considered by these authors.

respectively. It is also more reasonable to suppose that the reaction of the ions with the radicals  $R$  and  $R^1$  involve something like oxidation and reduction, as plausible mechanisms to explain the forcing of the reaction away from equilibrium.

In such a case, especially if  $n$  is moderately large, the criterion of greater resistance to diffusion per molecule to the right than to the left for the water pump would also probably be well satisfied. Due to their charge, ions have comparatively low diffusion constants. The diffusion constants of the species  $H_nR(OH)_n$  and  $Na_nRCl_n$  (or  $H_nR + R^1(OH)_n$  and  $Na_nR + R^1Cl_n$ ), diffusing in opposite directions, would be more or less similar, whereas the resistance to diffusion towards the right would be increased by the reciprocals of the low ionic diffusion constants.

The apparent marked specificity of some of the cases occurring in nature requires explanation. It is, of course, usual to expect in any reaction involving, say  $Na^+$ , that the replacement of this ion with  $K^+$  will not greatly change the equilibrium. Actually this apparent similarity of the two ions in ordinary reactions is somewhat overemphasized by the fact that in the majority of cases involving these ions we think of reactions which proceed practically completely in one or the other direction. In cases where the equilibrium constant of the reaction is close to unity, considerable difference in similar ions are observed, for instance, solubilities of potassium and sodium compounds are not usually so very closely the same. A difference of 10-fold in the value of the equilibrium constant for a chemical reaction is caused, at room temperature, by a difference of 1.4 kcal. in the free energy change, which is a very small absolute difference when one considers that bond energies are in the neighborhood of 30–80 kcal. In order that systems, such as those described here, should proceed without large dissipations of free energy, it is necessary that the reactions at the two surfaces proceed at close to equilibrium conditions. In such a case we could normally expect as much as a 10-fold difference in the equilibrium for, say  $Na^+$  and  $K^+$  ion, whereas much greater differences would hardly be remarkable.

If, in the case of a pump concentrating on ionic solute, the molecules schematized as  $b$ , carrying the ion to the left in combined form, carried 10 times as much  $K^+$  as  $Na^+$ , the former ion would be concentrated 10 times more than the latter. If there were a number of such units, working in series, especially if some with opposite specificity were

operating in opposite directions, almost complete separations of these two ions could be attained.

Finally, it might be mentioned that the later mathematical discussion shows that such pumps can only attain reasonably large thermodynamic efficiency if the concentration per stage is not too large. In some organs in which concentration is an important function, the morphology suggests that a number of different elements may work in series (as well as in parallel). This would seem to be an entirely probable arrangement, and would permit very great concentrations with relatively low energy output. In addition, as mentioned in the last paragraph, it would provide a simple means of obtaining very high specificity of effect.

We shall return now to the simple schematic representation of the single-cell pump, and show in detail how the necessary energy output may be computed.

### SOME GENERAL RELATIONS

In order to compute the ratio of expended free energy to that given to the transported material, we must introduce more symbols for the quantities involved.

We consider an apparatus like that described in the beginning of the previous section. We shall first consider processes by which solution or solvent can be pumped to higher hydrostatic pressures or lower osmotic pressures, respectively. Actually we shall consider both examples as one, and to simplify the discussion shall always speak only of the transfer of a pure solvent, presumably water, but whose properties will only be specified by giving  $\rho$ , the number of molecules per unit volume. The two membranes are at  $x=0$  and at  $x=l$  along the tube. The linear velocity of flow along the positive  $x$ -axis of the tube will be designated by  $v$ , which may be zero.

The cell may contain several solute molecular species,  $a, b, c$ , etc., whose concentrations, at any position in the cell, measured in molecules per unit volume will be designated by  $\rho_a, \rho_b, \rho_c$ , etc. The osmotic pressure of the solution is then  $kT(\rho_a + \rho_b + \dots)$ . We shall designate by  $\mu$  the chemical potential per molecule of the solvent (partial molal free energy divided by Avogadro's number), measured above that of the pure solvent at atmospheric pressure. If the external pressure on the solution is  $P$  above atmospheric we have:

$$\mu = \frac{1}{\rho} [P - kT(\rho_a + \rho_b + \dots)], \quad (1)$$

if we assume, as we shall, that the volume per molecule of the solvent remains constant.

We shall first investigate the forced flow of solvent through a cell containing  $\rho_a\%$  molecules of solute  $a$  per unit cross section, but with no chemical mechanism tending to maintain a concentration gradient. We assume that there is no friction to the flow

of solvent through the tube, or through the two semipermeable membranes. However, the membranes trap the solute molecules at a fixed position. The work done can be described as the work necessary to push, with the two membranes held a fixed distance  $l$  apart, the solute molecules through the solvent, at a velocity  $v$ .

If  $D_a$  is the diffusion constant of the solute molecules  $a$  in the solvent, the average retarding force is  $kTv/D_a$  per molecule. The distance they move in unit time is  $v$ . The product of force with the distance and with the number of molecules gives

$$W = \rho_a^0 kT v^2 l / D_a \quad (2)$$

as the work per unit cross-sectional area and unit time.

One may, however, derive the same result in an alternative but equivalent manner. The steady state equation for the concentration,  $\rho_a(x)$ , of solute  $a$  in a solution flowing with a velocity  $v$  along the positive  $x$ -axis is of the general form:

$$\rho_a(x) = (Q_a/v) + [\tau_a - (Q_a/v)] e^{x/D_a}, \quad (3)$$

in which  $\tau_a$  is the concentration at  $x = 0$ , and  $Q_a$  is the net transport in molecules per unit area and unit time, in the positive  $x$ -direction. The transport,  $Q_a$ , is zero in this case,  $\rho_a(x) = \tau_a \exp(vx/D_a)$ . The average density is fixed, and equal to  $\rho$ , and is

$$(1/l) \int_0^l \rho_a(x) dx = \tau_a (D_a/v) [\exp(vl/D_a) - 1].$$

We then have:

$$\tau_a = \rho_a^0 \frac{vl}{D_a} [e^{vl/D_a} - 1]^{-1}, \quad (4)$$

for the density at  $x = 0$ , and

$$\sigma_a = \rho_a^0 \frac{vl}{D_a} e^{vl/D_a} [e^{vl/D_a} - 1]^{-1}, \quad (5)$$

for the density at  $x = l$ . The difference is

$$\Delta\rho_a = \sigma_a - \tau_a = \rho_a^0 vl / D_a. \quad (6)$$

Now the osmotic pressure on the membrane at  $x = l$  is  $\sigma_a kT$ , and that at  $x = 0$  is  $\tau_a kT$ . The work necessary to move the two membranes (at a fixed distance apart) a distance  $v$  to the left in unit time is  $v(\sigma_a - \tau_a)kT$  per unit area of the membranes. Using (6) in this gives the result (2). We can thus calculate the work per unit time and cross-sectional area necessary to maintain the flow of velocity  $v$  as either due to the drag of the solute on the solvent, or interpret it as due to the difference in osmotic pressure on the two membranes.

For the general case that there is a transport of  $Q_a$  molecules per unit cross section and time of the molecules of solute  $a$  in the positive  $x$ -direction we may write, from (3),

$$\sigma_a = (Q_a/v) + [\tau_a - (Q_a/v)] e^{vl/D_a}, \quad (7)$$

as the concentration at the membrane  $x = l$  when the concentration is  $\tau_a$  at  $x = 0$ , or

$$\tau_a = (Q_a/v) + [\sigma_a - Q_a/v] e^{-vl/D_a}. \quad (8)$$

The net transport of solvent is  $Q = v\rho$ . The ratio  $Q_a/Q = Q_a/v\rho$  is the ratio of solute molecules of type  $a$  to solvent molecules which are transported in the positive

$x$ -direction. If we write (7) and (8) in terms of this ratio we find:

$$\sigma_a = \rho(Q_a/Q) + [\tau_a - \rho(Q_a/Q)]e^{Ql/D_a\rho}, \quad (7')$$

$$\tau_a = \rho(Q_a/Q) + [\sigma_a - \rho(Q_a/Q)]e^{-Ql/D_a\rho}. \quad (8')$$

The difference in density,  $\Delta\rho_a$ , of molecules  $a$  at the two ends  $x = l$  and  $x = 0$  is then  $\Delta\rho_a = \sigma_a - \tau_a = [\sigma_a - \rho(Q_a/Q)][1 - e^{-Ql/D_a\rho}] = [\tau_a - \rho(Q_a/Q)][e^{Ql/D_a\rho} - 1]$ . (9) In the limit that  $Q = 0$  this becomes

$$\Delta\rho_a = -Q_a l/D_a, \quad (9')$$

since  $1 - e^{-x} = x$  as  $x$  approaches zero.

If equation (9) is solved for  $Qa$  we find:

$$Qa = \frac{\tau_a}{\rho} Q \left\{ 1 - \frac{\Delta\rho_a}{\tau_a} (e^{Ql/D_a\rho} - 1)^{-1} \right\} = \frac{\sigma_a}{\rho} Q \left\{ 1 - \frac{\Delta\rho_a}{\sigma_a} (1 - e^{-Ql/D_a\rho})^{-1} \right\}, \quad (10)$$

as the transport of the solute  $a$  that is necessary to maintain the concentration difference  $\Delta\rho_a$  between the end at  $x = l$  and that at  $x = 0$ .

The difference in chemical potential of solute molecules at  $x = l$  minus that at  $x = 0$  is  $\Delta\mu_a = kT \ln(\sigma_a/\tau_a)$ , or

$$\Delta\mu_a = kT \ln \left[ 1 + \frac{\Delta\rho_a}{\tau_a} \right] = -kT \ln \left[ 1 - \frac{\Delta\rho_a}{\sigma_a} \right]. \quad (11)$$

### A HALF CELL PUMP

Now let us consider a simple case that, for obvious reasons, would not represent a satisfactory pump for continuous operation, but that illustrates the characteristics of the mechanism. Let us suppose that at the membrane to the left, at  $x = 0$ , a chemical reaction is capable of forming the solute  $a$  at a rate of  $Q_a$  molecules per unit area and time. The membrane at the right has the characteristic of absorbing this solute, and keeping its concentration in the solution at the interface at the constant value  $\sigma_a$ . There will then be a negative difference of concentration of  $a$ , the concentration at  $x = l$  being less than that at  $x = 0$ , by an amount  $\Delta\rho_a$ . If the membrane on the left is in equilibrium with pure solvent at atmospheric pressure in the range  $x < 0$ , that on the right will be in equilibrium with pure solvent at the higher pressure,  $\Delta P = -kT\Delta\rho_a$ , due to the difference in osmotic pressure at the two membranes. Alternatively the hydrostatic pressures may be equal but the concentrations of some solutes to which the membranes are not permeable may be lower on the right by an amount  $\Delta\rho_a$ . The solvent can now be made to flow from its lower to its higher chemical potential side by an infinitesimal increase in the rate of formation of solute at the left hand membrane. Neglecting the frictional forces of the tube and of the membranes, the flow velocity can be increased to any value by increasing this rate,  $Q_a$ , of production of solute on the left hand membrane.

The thermodynamic efficiency of the pump is easy to compute. The increase in chemical potential of the solvent per molecule is  $\Delta P/\rho = -kT\Delta\rho_a/\rho$ , and with the transport  $Q$  is:

$$\Delta F \text{ solvent per cm}^2 \cdot \text{sec} = \frac{Q\Delta P}{\rho} = -\frac{QkT}{\rho} \Delta\rho_a. \quad (12)$$

The decrease in free energy of the solute in passing from the membrane at  $x = 0$  to that at  $x = l$  is given by the product of (10), which gives the transport, and the negative of (11), which gives the chemical potential decrease per molecule:

$$-\Delta F \text{ solute per cm}^2 \cdot \text{sec} = \frac{QkT}{\rho} \left[ \sigma_a - \Delta\rho_a(1 - e^{-Ql/D_a\rho})^{-1} \right] \ln \left( 1 - \frac{\Delta\rho_a}{\sigma_a} \right). \quad (13)$$

The ratio,  $R$ , of (13) to (12) gives

$$R = \left[ -\frac{\sigma_a}{\Delta\rho_a} + (1 - e^{-Ql/D_a\rho})^{-1} \right] \ln \left( 1 - \frac{\Delta\rho_a}{\sigma_a} \right). \quad (14)$$

as the ratio of free energy expended to that gained as increase in the free energy of the solvent. If  $Ql/D_a$  is large we find:

$$R = \left( 1 + \frac{1}{x} \right) \ln(1 + x),$$

where  $x = -\Delta\rho_a/\sigma_a = (\tau_a - \sigma_a)/\tau_a > 0$ . This ratio is always greater than unity, and has values:

$x = 0.01$	0.05	0.1	0.25	0.5	1
$R = 1.005$	1.025	1.048	1.115	1.216	1.386

If  $Ql/D_a$  is small, the ratio  $R$  does not approach as close to unity. We see, for instance, that if the cell contained ionic NaCl at 0.1  $M$ , and  $\Delta\rho = 0.01/N_0$ , the increase in osmotic pressure would be almost a half atmosphere, and one could approach within about 5% of full thermodynamic efficiency were there negligible friction in the membranes.

This pump can hardly represent a satisfactory model, in spite of its efficiency, since it has merely thrown the difficulty one stage back. In order to complete the cycle we must remove the solute,  $a$ , from the membrane at the right, and raise its chemical potential sufficiently to supply it to the membrane on the left. We shall now discuss a more completely self contained unit that requires only the supply of free energy rather than the retransfer of material from low to higher potential.

### THE COMPLETE UNIT

The simplest complete unit would consist of a cell containing two kinds of molecules,  $a$  and  $b$ , with the possible chemical reaction:



We assume that this associative reaction cannot take place in solution, but is catalyzed enzymatically by the membrane on the right to equilibrium at the concentrations,  $\sigma_a$ , and  $\sigma_b$ , of  $a$  and  $b$ , respectively. At whatever concentrations,  $\tau_a$  and  $\tau_b$ , the molecules are at the left hand membrane, a reaction supplying free energy is able to dissociate  $b$  and  $a$ , forming  $Q_a$  molecules of  $a$  per unit area and time. The flow of  $b$  must then be in the direction of the negative  $x$ -axis, and given by

$$Q_b = -Q_a/n. \quad (16)$$

We suppose the reaction on the left adjusts the energy supply to maintain the



total (negative) concentration difference:

$$\Delta\rho = \Delta\rho_a + \Delta\rho_b = \sigma_a + \sigma_b - \tau_a - \tau_b = [\sigma_a - \rho(Q_a/Q)][1 - e^{-Q/D_a\rho}] + [\sigma_b + \rho(Q_b/nQ)][1 - e^{-Q/D_b\rho}], \quad (17)$$

from (9) with (16). If we use  $\Delta\rho_a$  for the first of these expressions, and replace  $Q_a$  from (10) in the second, we obtain an expression from which we can compute  $\Delta\rho_a$  in terms of  $\Delta\rho$ ,  $\sigma_a$ , and  $\sigma_b$ . By setting  $\Delta\rho_b = \Delta\rho - \Delta\rho_a$ , we find the value of  $\Delta\rho_b$ . The transport,  $Q_a$ , from (10), can also be expressed in terms of  $\Delta\rho$ ,  $\sigma_a$ , and  $\sigma_b$ . The algebra is moderately tedious, but not complicated. Using

$$A = 1 - e^{-Q/D_a\rho}, \quad (18)$$

$$B = \frac{1}{n} (1 - e^{-Q/D_b\rho}), \quad (19)$$

we find:

$$\rho \frac{Q_a}{Q} = \frac{1}{A-B} [n\sigma_b B + \sigma_a A - \Delta\rho], \quad (20)$$

$$1 - \frac{\Delta\rho_a}{\sigma_a} = 1 + \frac{AB}{A-B} \left( \frac{n\sigma_b}{\sigma_a} + 1 \right) - \frac{A}{A-B} \frac{\Delta\rho}{\sigma_a}, \quad (21)$$

$$1 - \frac{\Delta\rho_b}{\sigma_b} = 1 - \frac{\sigma_a}{\sigma_b} \frac{AB}{A-B} \left( \frac{n\sigma_b}{\sigma_a} + 1 \right) + \frac{\sigma_a}{\sigma_b} \frac{B}{A-B} \frac{\Delta\rho}{\sigma_a}. \quad (22)$$

The change in chemical potential of the solvent is  $-kT\Delta\rho/\rho$  per molecule, and this multiplied by the transport,  $Q$ , gives the increase in free energy of the solvent per unit cross-sectional area and time. The difference in chemical potentials of the solute species  $a$  and  $b$  are  $kT \ln[1 - (\Delta\rho_a/\sigma_a)]$  and  $kT \ln[1 - (\Delta\rho_b/\sigma_b)]$  respectively at the two membranes. We assume that the reaction on the right,  $x = l$ , goes at equilibrium, so the free energy,

$$\Delta\mu = kT \left\{ \ln \left( 1 - \frac{\Delta\rho_a}{\sigma_a} \right) - \frac{1}{n} \ln \left( 1 - \frac{\Delta\rho_b}{\sigma_b} \right) \right\} \quad (23)$$

must be supplied at the left hand membrane per molecule of  $a$  formed. If this is multiplied by  $Q_a$ , the transport, one finds the free energy which must be supplied per unit area of membrane and unit time. The ratio,  $R$ , of free energy supplied to that given to the solvent is then:

$$R = - \left( \rho \frac{Q_a}{Q} \right) \frac{1}{\Delta\rho} \left\{ \ln \left( 1 - \frac{\Delta\rho_a}{\sigma_a} \right) - \frac{1}{n} \ln \left( 1 - \frac{\Delta\rho_b}{\sigma_b} \right) \right\}. \quad (24)$$

If we introduce

$$n\sigma_b/\sigma_a = y, \quad (25)$$

$$-\Delta\rho/\sigma_a = x, \quad (26)$$

and use (20), (21), and (22) in (24), we find:

$$R = \frac{1}{A-B} \left[ 1 + \frac{A}{x} + \frac{By}{x} \right] \left\{ \ln \left[ 1 + \frac{AB}{A-B} (y+1) + \frac{A}{A-B} x \right] - \frac{1}{n} \ln \left[ 1 - \frac{n}{y} \frac{AB}{A-B} (y+1) - \frac{n}{y} \frac{B}{A-B} x \right] \right\}. \quad (27)$$

This rather complicated expression can be simplified if the quantities  $\Delta\rho_a/\sigma_a$  and

$\Delta\rho_b/\sigma_b$  are both small, since  $\ln(1+x) \cong x$  for small values of  $x$ . This will be the case if  $A$ ,  $B$  and  $x$  are all very small. In this case one finds

$$R \cong \frac{1}{(A-B)^2} \left[ 1 + \frac{A}{x} + \frac{By}{x} \right] \left[ \frac{AB}{y} (y+1)^2 + x.1 + \frac{Br}{y} \right]. \quad (28)$$

For small values of  $A$  and  $B$ , equations (18) and (19), these are

$$A = Ql/D_a\rho, \quad (29)$$

$$B = Ql/D_b\rho n. \quad (30)$$

One may expect there would be a value of the flow  $Q$  for which  $R$  would have a minimum value, since when there is no flow the value of  $R$  is obviously infinity, whereas if the flow is too rapid the molecules of type  $b$  cannot diffuse back against the stream to  $x = 0$ . We may try to set

$$\alpha = B/A = D_a/nD_b \quad (31)$$

and

$$Z = \frac{r}{A} = \frac{\Delta\rho}{\sigma_a} \frac{D_a\rho}{Ql}, \quad (32)$$

in (24), obtaining

$$R = \frac{\alpha}{(1-\alpha)^2} \frac{(y+1)^2}{y} \left[ 1 + \frac{1}{Z} (1 + \alpha y) \right] \left[ 1 + Z \frac{y + \alpha}{\alpha(y+1)^2} \right], \quad (33)$$

and try to find  $Z_m$  for which  $R$  is a minimum. One finds:

$$Z_m = (1+y) \sqrt{\alpha(1+\alpha y)/(y+\alpha)}. \quad (34)$$

Using this in  $R$  one has

$$R_{\min} = K^{-1} [1 + \sqrt{1+K}]^2, \quad (35)$$

where

$$K = \frac{(1-\alpha)^2}{\alpha} \frac{y}{(y+1)^2}. \quad (36)$$

Now seeking the value of  $y$  for which  $R_{\min}$  has its lowest value, we find it at  $y = 1$ .

The final result is then for the double minimum with optimum  $Z$  and  $y$  that:

$$R_{\min} = \frac{4\alpha}{(1-\alpha)^2} \left[ 1 + \sqrt{1 + \frac{(1-\alpha)^2}{4\alpha}} \right]^2, \quad (37)$$

where  $\alpha$ , given by equation (31), is the ratio of  $D_a$ , the diffusion constant of the solute  $a$ , to  $n$  times  $D_b$ , the diffusion constant of the associated solute. This efficiency is attained when  $y = 1$ , which means that (equation (25)) the concentration of the associated form  $b$  at the right hand membrane is  $1/n$  times that of the solute  $a$ . The flow for which this efficiency is attained is obtained from (34) with  $y = 1$  and (32), namely:

$$Q = \frac{\Delta\rho}{\sigma_a} \frac{D_a\rho}{l} \frac{1}{2\sqrt{\alpha}}, \quad v = \frac{Q}{\rho} = \frac{\Delta\rho}{\sigma_a} \frac{D_a}{l} \frac{1}{2\sqrt{\alpha}}. \quad (38)$$

The equation becomes invalid as  $\alpha$  approaches unity too closely, since then the development of the logarithm would have been unjustified. The flow would be to the right, as supposed in the discussion, if  $\alpha < 1$ , and in the reverse direction if  $\alpha > 1$ .

The value of  $R_{\min}$  for some values of  $\alpha$  is:

$\alpha =$	0.1	0.25	0.5
$R_{\min} =$	3.03	9	34

The diffusion constant  $D$  is approximately proportional to the reciprocal of the radius of a molecule. We might assume that the radius of an associated complex is approximately  $n^{\frac{1}{2}}$  times that of the dissociated molecules. We could then estimate reasonable values of  $D_b$  as about  $n^{\frac{1}{2}}$  times  $D_a$  so that  $\alpha$  would be about  $n^{-1}$  (equation (31)). If  $n$  were fairly large one might attain quite respectable efficiencies for such a cell as that described here.

Diffusion constants are of the order of magnitude of  $10^{-5}$  cm<sup>2</sup>/sec. With equation (38) assuming  $\Delta\rho/\sigma_a$  about 0.1, and cell thickness  $l = 0.01$  mm. one finds the optimum flow velocity to be about  $10^{-3}$  cm./sec. or approximately 1 mm./min.

### THE SOLUTE DIFFUSION PUMP

We have discussed only the pumping of solvent. Let us now investigate the diffusion concentration of the solute and assume that the membranes of the cell previously described are permeable to solute  $a$ , but not to the associated form  $b$ . Let us then consider the case that there is no net flow of solvent,  $v = 0$ ,  $Q = 0$ . The solutions on right and left of the cell would be in equilibrium with the cell if both had hydrostatic pressures differing by  $kT\Delta\rho_b$ , and concentrations  $\sigma_a$  and  $\tau_a = \sigma_a - \Delta\rho_a$  of solute  $a$  respectively. With a slight increase in concentration of solute on the right there would be a tendency for it to diffuse through the cell to the left hand side of greater concentration.

For this case we must use equation (9') for the limit  $Q = 0$ , that

$$\Delta\rho_a = -Q_a l/D_a, \quad (39)$$

$$\Delta\rho_b = -Q_b l/D_b. \quad (40)$$

If (compare with equation (16))

$$Q_a = -nQ_b - Q^*, \quad (41)$$

then there will be a net transport,  $Q^*$ , of solute  $a$  to the left. Using  $\alpha$ , equation (31), and (41) in (40),

$$\begin{aligned} \Delta\rho_b &= (Q_a + Q^*)l/nD_b \\ &= \alpha(Q_a + Q^*)l/D_a \\ &= -\alpha[1 + (Q^*/Q_a)]\Delta\rho_a. \end{aligned} \quad (42)$$

The free energy supplied to the reaction per molecule  $a$  liberated at the left is given by (23). The number of molecules liberated is  $Q_a + Q^*$  per unit area of membrane and unit time. The increase in chemical potential of  $a$  in passing from the right to the left is  $\Delta\mu_a = kT \ln[1 - (\Delta\rho_a/\sigma_a)]$ , and the net transport is  $Q^*$ . The ratio of expended free energy to that given to the solute molecules which diffuse from right to left is:

$$\begin{aligned} R &= \frac{(Q_a + Q^*) \left\{ \ln [1 - (\Delta\rho_a/\sigma_a)] - \frac{1}{n} \ln [1 - (\Delta\rho_b/\sigma_b)] \right\}}{Q^* \ln [1 - (\Delta\rho_a/\sigma_a)]} \\ &= [1 + (Q_a/Q^*)] \left\{ 1 - \frac{\frac{1}{n} \ln [1 - (\Delta\rho_b/\sigma_b)]}{\ln [1 - (\Delta\rho_a/\sigma_a)]} \right\}. \end{aligned} \quad (43)$$

If  $\Delta\rho_a/\sigma_a$  and  $\Delta\rho_b/\sigma_b$  are both small, we may, as before, approximate  $\ln(1-x)$  with  $-x$ , obtaining

$$R = [1 + (Q_a/Q^*)] \left[ 1 - \frac{1}{n} \frac{\Delta\rho_b\sigma_a}{\Delta\rho_a\sigma_b} \right],$$

and with (42), and with (25) for  $y = n\sigma_b/\sigma_a$ ,

$$\begin{aligned} R &= [1 + (Q_a/Q^*)] \left\{ 1 + \frac{\alpha}{y} [1 + (Q'/Q_a)] \right\} \\ &= [2 + \zeta^{-1} + \zeta] \left[ 1 + \frac{\alpha}{y} \right] - 1, \end{aligned} \quad (44)$$

if

$$\zeta^{-1} = Q^*/Q_a, \quad (45)$$

is the ratio of the net transport (towards the left) to the actual transport (towards the right) of the solute  $a$  in the cell. The ratio  $\zeta$  is more appropriately expressed from (39) as

$$\zeta = - \frac{\Delta\rho_a D_a}{l Q^*}, \quad (46)$$

and is seen, by comparison with (32), to be strictly analogous to  $Z$  in the previous example.

The ratio of expended to useful free energy,  $R$ , has a minimum when  $\zeta = 1$ , or

$$Q^* = \Delta\rho_a \frac{D_a}{l}. \quad (47)$$

For low values of  $\alpha$  and high values of  $y$  this ratio of expended to gained free energies can approach values as low as 3.

## SUMMARY

An osmotic diffusion pump is described which can use chemical energy to transport materials from one solution to another in the direction opposite to the natural diffusion tendency. Essentially the same mechanism could pump either solute or solvent. It is suggested that this may be the mechanism used in living tissues for this purpose.

The scheme is to utilize the cross-current diffusion of the reactants and products of a chemical reaction, formed catalytically at one membrane wall, and reversed in excess of equilibrium at the other wall. This reaction is assumed to be driven by some other chemical reaction which releases the necessary free energy.

It is shown that under favorable circumstances the ratio of expended free energy to that given to the transported material may be as low as about three.



# A Comparison of Three Thiochrome Methods for Urinary Thiamine by a Simplified Base-Exchange Procedure <sup>1</sup>

Evangeline Papageorge and Margaret Vogt Lamar

*From the Department of Biochemistry, Emory University, Georgia*

Received August 23, 1946

## INTRODUCTION

One great advantage of thiochrome methods for the determination of thiamine in urine, is their great sensitivity which permits the use of much smaller samples than do the colorimetric procedures based upon the Ehrlich-Pauly reaction. The presence of interfering material in urine invalidates assay by direct oxidation to thiochrome and necessitates a preliminary base-exchange operation for the isolation of thiamine, such as that prescribed in the Hennessy and Cerecedo method (4, 5). That this does not solve all the difficulties is evidenced by the proposal of several techniques (7, 9, 10 and 13) for the oxidation and final extraction steps in attempts to remove or correct for non-thiochrome material which may persist after elution and which is apparently affected in the oxidation step, thus invalidating the Hennessy-Cerecedo alkali blank.

This paper is concerned with the presentation of a simple and rapid base-exchange procedure and its application to three modifications of the Hennessy and Cerecedo method<sup>2</sup> (4, 5). Adsorption, elution, oxidation and extraction are all carried out successively in the same vessel, a glass-stoppered tube with conical bottom. After separation and clarification of the isobutanol solution of thiochrome, one is ready for fluorometric assay. No adsorption columns are required, thus avoiding

<sup>1</sup> Aided by grants from the University Center in Georgia and The Winthrop Chemical Company.

Originally presented before the Division of Biological Chemistry at the Atlantic City Meeting of the American Chemical Society, April, 1946.

<sup>2</sup> The Hennessy and Cerecedo method was based essentially upon that originated by B. C. P. Jansen, *Rec. trav. chim.*, **55**, 1046 (1936).

the inconvenience involved whenever these become clogged, and also eliminating the need for suction apparatus, which is a problem in small laboratories whenever a large number of thiamine analyses are to be made. Permutit has been advantageously substituted for Decalso, rendering centrifugation unnecessary and loss in decantation minimal.

Our technique does not differ fundamentally from that of Johnson, Sargent *et al.* (6) although it was developed entirely independently. However, since there are some differences in technique, details are given under "Base-Exchange" below. Perlzweig, Kamin *et al.* (12) have also published a rapid technique similar in some respects to that of Johnson *et al.* and to ours. However, they employ superfiltrol for adsorption and this necessitates the use of an acid pyridine-methanol solution as an eluant. We prefer acid potassium chloride for this purpose since it is more pleasant to use and less expensive.

After testing the simplified technique with standard solutions it was applied to the determination of thiamine in urine according to the Merck and Co., Inc., adaptation of the Hennessy and Cerecedo method (8). Calculations were made according to Najjar and Ketron (10), who advocate the use of a correction factor for the reduction of fluorescence in the isobutanol solution of thiochrome caused by the oxidation of  $F_2$ .  $F_2$  is  $N^1$ -methylnicotinamide, a niacin metabolite, which persists in the eluate and which may be present in sufficient concentration to cause major errors in the thiamine values as determined by the Hennessy and Cerecedo method.

The simplified technique was then applied to two other modifications of the Hennessy method: a fluorometric adaptation of the Urban and Goldman procedure (13), and the method of Mickelsen, Condiff and Keys (9). Urban and Goldman recommend the use of benzenesulfonyl chloride for the destruction of thiamine in the blank determination of non-thiochrome blue-fluorescent material present in the isobutanol extract. Mickelsen and his coworkers remove most of these interfering substances by suitable adjustment of pH at the isobutanol extraction stage. In preliminary experiments, sodium sulfite was also employed for the destruction of thiamine in the blank determination (7, 12) but since the results were not always reliable, the use of sodium sulfite blanks was discontinued.

Comparative determinations were made with each of the three thiochrome methods referred to above using the Hennessy base-exchange procedure (4) and our modified technique. Recovery checks

were made with most of the determinations by adding 1  $\gamma$  of thiamine to 2 ml. of urine and submitting this to the same total procedure as the analogous sample without added thiamine. Where the urine specimen had a high concentration of thiamine, 1  $\gamma$  of the vitamin was added to only 1 ml. of urine in the recovery check.

## EXPERIMENTAL

### *Reagents*

1. Glacial acetic acid (C.P.)
2. Activated permutit. About 700 ml. of a 25% KCl-5% acetic acid solution are added to 100 g. of permutit. The mixture is boiled for one hour with constant stirring (mechanical stirrer). The supernatant is discarded and the treatment with KCl-acetic acid is repeated. The permutit is then washed with boiling distilled water until chloride-free. It is dried at about 100°C. and stored dry. The activation process should be carried out once a month.
3. Thiamine standards (Perlzweig *et al.* (12)).
  - (a) The stock solution contains 0.100 mg. of thiamine chloride/ml. and is made up in 0.05 *N* HCl. It is prepared every 3 months and is stored in an amber bottle in the refrigerator.
  - (b) The working standard containing 1.00  $\gamma$ /ml. is prepared weekly by dilution of 1 ml. of stock solution to 100 ml. with 0.05 *N* HCl. It is stored in an amber bottle in the refrigerator.
4. 2% KCl (C.P.) in approximately 0.1 *N* HCl.
5. 2% potassium ferrieyanide (C.P.). This is prepared weekly and kept in a dark bottle.
6. 15% NaOH (C.P.).
7. Alkaline ferrieyanide. This consists of 1 ml. of reagent 5 mixed with 29 ml. of reagent 6. This mixed reagent is said to keep only four hours.
8. Isobutanol, free from fluorescent material. Each batch should be tested for fluorescence. It may be recovered after use by redistillation or by treatment with activated charcoal (11) as follows: 1 liter of isobutanol is shaken with 50 g. of activated charcoal (Darco-G-60) for 10 minutes and then filtered three times to remove the charcoal completely.
9. Benzenesulfonyl chloride (Eastman).
10. Acid mixture for pH adjustment (9). This is a mixture of equal volumes of concentrated HCl and 85%  $\text{H}_2\text{PO}_4$ .
11. Preheated anhydrous sodium sulfate (C.P.). The reagent is freed from fluorescent material by heating at 800°–1000°F. in a muffle furnace for several hours (1). A large batch of  $\text{Na}_2\text{SO}_4$  may be so treated and stored.

### *Special Apparatus*

1. Conical shape centrifuge tubes with ground-glass stoppers and of at least 30 ml. capacity. Pyrex tubes of 40–50 ml. capacity were made for us by E. Machlett and Son, 233 E. 23rd St., New York 10, New York.



2. Small Squibb separatory funnels with glass stoppers. Funnels of 30 ml. capacity serve well.
3. Fluorophotometer. A model B Pfaltz and Bauer instrument was used in the work described in this paper. The Coleman photofluorometer (Model 12) is said to be more sensitive but was not available to us.

### *Preservation of Urine*

The addition of 2 ml. of glacial acetic acid per 100 ml. of urine and refrigeration of the acidified specimen preserves the thiamine content for at least one month (2). The treatment with acetic acid also usually results in a pH of 4.0-5.0 which is necessary for proper base-exchange. However, it is wise to check the pH before beginning the adsorption procedure.

### *Base-Exchange (Adsorption and Elution)*

Place 300 mg. of activated permutit (horn-balance) into each of three special centrifuge tubes, two of which are for duplicate analysis and the third for blank determination. Add to each tube 2-5 ml. of urine depending upon the concentration of thiamine. The amount of thiamine in the aliquot taken for analysis should preferably not exceed 1.2  $\gamma$ . Dilute to 10 ml. with distilled water, stopper and shake for 3 minutes. Remove stoppers carefully and wash permutit from each into its tube with water, simultaneously washing down any permutit clinging to the sides of the tube. If there has been much foaming add 1-2 drops of caprylic alcohol. Let settle and decant, discarding supernatant. Add 10 ml. of water and repeat the operation. To each tube now add 5 ml. of acid KCl, stopper and shake for 3 minutes. This completes the adsorption and elution. The temperature of the KCl solution appears to have no effect on the extent of elution as has been observed by Friedemann and Kmiecik (3).

Oxidation to thiochrome and extraction with isobutanol may now be carried out in the presence of the permutit according to any of the procedures described below.

### *Oxidation and Extraction*

(1) *Modification of Najjar-Ketron Procedure.* Add 15 ml. of isobutanol to all three tubes and then add 3 ml. of NaOH to the blank and 3 ml. of alkaline ferricyanide to each of the other two. Stopper and shake immediately for exactly 1.5 minutes. Let stand for 10 minutes to allow the permutit to settle out and the layers to separate. Transfer to separatory funnels taking care that no permutit enters the funnel. Remove and discard the aqueous layer. Add 1 level scoopful of  $\text{Na}_2\text{SO}_4$  (about 2 g.), stopper and shake until clear. Transfer carefully to small brown bottles without permitting any of the sodium sulfate to accompany the liquid. Stopper and store in refrigerator until fluorescence is determined. The thiochrome solutions keep overnight even at room temperature but refrigeration ensures preservation for at least 24 hours.

(2) *Modification of Urban-Goldman Procedure* (benzenesulfonyl chloride blank). Treat the two tubes for oxidation to thiochrome as above. To the blank add 3 ml. of 15% NaOH and 1 drop of benzenesulfonyl chloride. Shake for 3 minutes. Add 0.1 ml. of 2% ferricyanide and let stand 1 minute. Add 15 ml. of isobutanol and shake for 1.5 minutes. Proceed as above for separation of layers, clearing of isobutanol solution, etc.

(3) *Modification of Michelsen, Conditt and Keys Procedure* (adjustment of pH before extraction of isobutanol). Add 3 ml. of NaOH to the blank and 3 ml. of alkaline ferricyanide to each of the other two tubes. Stopper and shake for exactly 1.5 minutes. Adjust to pH 8.0-10.0 by the addition of 0.45-0.55 ml. of HCl-H<sub>3</sub>PO<sub>4</sub> mixture. The end-point is indicated by the formation of a white precipitate. The exact amount of acid-mixture necessary to make the pH adjustment can be determined by checking the pH of the thiochrome solutions with a pH meter every time a new batch of acid KCl, 15% NaOH or HCl-H<sub>3</sub>PO<sub>4</sub> is prepared. As soon as the pH is adjusted, cool tubes in running water, as addition of the strong acid mixture results in the liberation of heat, and add 15 ml. of isobutanol to each tube. Shake for 1.5 minutes and proceed as above for the rest of the operation.

### *Thiochrome Standards and Standard Blank*

These may be prepared for the standardization of the fluorophotometer without preliminary adsorption and elution since standard solutions submitted to the whole procedure give 96-100% of the values obtained by direct oxidation. A set of standards should be freshly prepared every time a series of fluorometric readings are made. Place 1 ml. of dilute standard (contains 1.00  $\gamma$  of thiamine) into each of three Squibb separatory funnels (Machlett thiamine reaction vessels with glass stoppers and stopcocks serve even better but these are more expensive). To each add 4 ml. of acid KCl and 15 ml. of isobutanol. To the blank add 3 ml. of NaOH and to each of the other two 3 ml. of alkaline ferricyanide. Stopper and shake immediately for exactly 1.5 minutes. Let stand 10 minutes and remove aqueous layer. Add 1 level scoopful of Na<sub>2</sub>SO<sub>4</sub> to each isobutanol extract, stopper and shake until clear. Pour into small brown bottles and store in the refrigerator until fluorometric determinations are made.

### *Fluorometric Assay*

The iris diaphragm in our instrument was set at 40 so that the standard prepared from 1  $\gamma$  of thiamine produced a deflection of 50-60 galvanometer units. Quinine sulfate standards were not used since thiochrome standards and blank were prepared for each series of determinations. A standard thiochrome block was employed to check the light intensity during the period of fluorometric readings. Fluctuations in voltage may occur so suddenly, however, that corrections cannot be made properly for alterations in light intensity. The only solution to this problem is an instrument with a reliable voltage stabilizer.

### *Calculations*

The micrograms of thiamine found in the sample taken for analysis may be calculated from the equations below. The following symbols indicate galvanometer readings:

*U* = unknown

*S* = standard

*A* = alkali blank

*B* = standard blank

*BSCl* = benzenesulfonyl chloride blank

1. Najjar-Ketron calculation (10):

$$\frac{(U-B) - 0.2(A-B)}{S-B}$$

2. Benzenesulfonyl chloride blank:

$$\frac{U - BSCl}{S - B}$$

3. pH adjustment:

$$\frac{U - A}{S - B}$$

## RESULTS AND DISCUSSION

Table I gives comparative values for urinary thiamine found by the three thiochrome methods as adapted to our simplified procedure and

TABLE I  
*Comparative Values for Urinary Thiamine Found by Adaptation of the Simplified Procedure to Three Thiochrome Methods and by Customary Base-Exchange Employing Adsorption Columns*  
(Thiamine values are given in  $\gamma$ /ml. of urine)

Urine Sample No.	Simplified Procedure			Customary Base-Exchange		
	N-K <sup>1</sup>	U-G <sup>2</sup>	M-C-K <sup>3</sup>	N-K <sup>1</sup>	U-G <sup>2</sup>	M-C-K <sup>3</sup>
1	0.10	0.09	0.08	0.15	0.12	0.12
2	0.03	0.02	0.02	0.04	0.04	0.04
3	0.23	0.21	0.21	0.24	0.24	0.22
4	0.13	0.12	0.12	0.16	0.15	0.14
5	0.11	0.11	0.10	0.12	0.13	0.11
6	0.50	0.47	0.41	0.47	0.47	0.47
7	0.31	0.29	0.29	0.35		0.35
8	0.36		0.33	0.40		0.35
9	0.10	0.10	0.10	0.13		0.12
10	0.26	0.25	0.23	0.27		0.23
11	0.05	0.05	0.04	0.05		0.05
12	0.17	0.16	0.16			
13	0.32	0.32	0.28			
14	0.14	0.13	0.12			
15	0.26	0.29	0.27			
16	0.03	0.04	0.04			
17	0.07	0.08	0.06			

<sup>1</sup> Najjar-Ketron Method.<sup>2</sup> Fluorometric Adaptation of Urban and Goldman Method.<sup>3</sup> Method of Mickelsen, Condiff and Keys.

as carried out with the usual technique employing adsorption columns. Table II shows the recoveries obtained on some of the same urines by use of the simplified and the usual base-exchange procedures with the three methods.

TABLE II

*Per Cent of Added Thiamine Recovered Upon Addition of 1  $\gamma$  of Thiamine to 2 ml. of Urine as Found by Adaptation of Simplified Procedure to Three Thiochrome Methods and by Usual Base-Exchange Employing Adsorption Columns*

(Urine sample numbers correspond to those in Table I which gives thiamine content of samples involved)

Urine Sample No	Najjar-Ketron		Urban-Goldman		Mickelsen <i>et al</i>	
	Simplified	Usual	Simplified	Usual	Simplified	Usual
	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
1	98	101	96	100	84	86
2	88	87	85	88	76	80
3	98	93	94	92	90	90
4	90	94	89	94	87	84
5	99	94	100	94	91	82
6	89	107	89	105	93	107
7	95	90			96	83
8	86	104			92	105
9	94	84			96	82
10	97	104			95	86
11	91	83			82	78
Mean	93	95	92	96	89	88
$S_D^1$	2.88		3.35		3.48	

<sup>1</sup>  $S_D$  = standard error of the difference between the means.

Agreement between the two base-exchange techniques is good. In some specimens containing larger concentrations of thiamine than 0.25  $\gamma$ /ml., we found better checks between the two base-exchange procedures if only 2 ml. of urine were used for analysis by the simplified technique. We recommend, therefore, that 2 ml. of urine be used routinely. If extremely low values are obtained the analysis can be repeated with larger volumes of the specimen. However, since urines low in thiamine frequently show poor recovery (2, 9), apparently because the ratio of interfering material to thiamine is high, it appears inadvisable to increase the volume taken for analysis beyond 5 ml.

The *per cent* of added thiamine recovered by our technique also compares well with that recovered by the usual procedures. The equation for small samples<sup>3</sup> was used to calculate  $S_D$ , the standard error of the difference between the means. The  $S_D$  values indicate no significant difference between the mean *per cent* recovery found by the two base exchange procedures.

Comparison of urine thiamine content as determined by the application of our simplified technique to the three thiochrome methods shows excellent agreement in general. The modified Mickelsen-Condif-Keys procedure most frequently gave the lowest values but an appreciable discrepancy was observed in only 1 instance, that of sample No. 6, in which the difference between extreme values was 0.09  $\gamma$  thiamine/ml. of urine. The next greatest difference was 0.04  $\gamma$  in sample No. 13. Values for all other urines agreed within 0.03  $\gamma$ /ml. as determined by all three methods.

Recovery checks, some of which are shown in Table II likewise showed no significant variation between any of the three methods employed. In 39 comparative analyses (13 urines) by our adaptation of the three procedures, recoveries ranged from 76 to 105%. The mean *per cent* recovery found in 13 urines by application of the simplified base-exchange technique was as follows: Najjar-Ketron, 96%; Urban-Goldman, 94%; Mickelsen-Condif-Keys, 92%. The standard error of the difference between the two extreme means of *per cent* recovery, 92 and 96%, is 2.63 which indicates that the difference of 4% is not significant.

Thus, on the basis of our findings, the three methods studied appear to be equally accurate. None of our results show as marked discrepancies as those observed by Mickelsen and his coworkers between values found by their method and those by the method of Najjar and Ketron which the Minnesota workers say gives erroneously high results. Mickelsen and his associates also found a variable ratio between thiamine blank and  $F_2$  excretion in high blank urines. Only one of the specimens analyzed in our comparative series had an unusually high blank. This was specimen No. 6 for which the same thiamine value was found by all three methods when adsorption columns were

$$S_D = \frac{\sqrt{\frac{\Sigma(x_1^2) + \Sigma(x_2^2)}{(N_1-1) + (N_2-1)}}}{\sqrt{\frac{N_1 N_2}{N_1 + N_2}}}$$

employed. When our base-exchange technique was used, the Mickelsen-Condif-Keys value was 0.09  $\gamma$  less than the Najjar-Ketron figure and 0.06  $\gamma$  less than the value found with the benzenesulfonyl chloride blank. On the other hand, urine No. 4, which was a mixture of equal parts of the low thiamine sample No. 2 and of sample No. 3, gave thiamine values by all procedures which agree well with the average calculated from the corresponding values for samples 2 and 3.

In a single experiment we added 5  $\gamma$  of  $F_2$ /ml. of urine to each of two specimens not included in the above tables, one of which had a high blank and the other a low blank value. Determination of the thiamine content by our modification of the Najjar-Ketron method (simplified base-exchange) showed no effect of added  $F_2$  on the thiamine content of these specimens, although the alkali blank was markedly increased by the addition of  $F_2$ .

Our data are not sufficient to show whether or not the Najjar-Ketron method is always reliable. Nor do we have enough evidence either for or against the accuracy of the benzenesulfonyl chloride blank on the grounds discussed by Mickelsen, Condif and Keys for the former method. The use of the benzenesulfonyl chloride blank is more convenient than the procedure involving pH adjustment and it is not open to the criticism involving correction for  $F_2$ . The findings of Urban and Goldman (13) indicate that benzenesulfonyl chloride has little if any effect on the non-thiochrome fluorescent material present in the oxidized eluate. Further study is necessary, however, to indicate which of the three methods is the most consistently reliable.

#### ACKNOWLEDGMENTS

The authors wish to acknowledge the technical assistance of Miss Alice Rayle and to express their gratitude to Dr. George T. Lewis for his interest and invaluable criticism in this study

#### SUMMARY

1. A simplified base-exchange technique which does not require the use of adsorption columns is described. This gives results comparable to those found by the customary procedure for adsorption and elution in which permutit columns are employed. Our technique results in considerable saving of time since the analyst can complete about twice as many analyses in a given working period.

2. The rapid technique was adapted to three thiochrome methods for the determination of thiamine in urine. Agreement between the three was generally good. Slightly lower values were obtained in the method involving adjustment to pH of 8.0–10.0 before extraction of the thiochrome with isobutanol but the differences were not appreciable. Recovery checks showed no significant variation between any of the three methods employed.

3. Further investigation is necessary to indicate which of the three methods studied is the most consistently reliable. The use of benzene-sulfonyl chloride for the specific destruction of thiamine offers the most convenient method for the determination of non-thiochrome fluorescent material in the isobutanol extract.

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# On the Inactivation of Estradiol by Rat Liver *in Vitro*<sup>1,2</sup>

Hilton Levy<sup>3</sup>

*From the Laboratories of Memorial Hospital for Cancer and Allied  
Diseases, N. Y. C., and the Department of Chemistry, Polytechnic  
Institute of Brooklyn, Brooklyn, N. Y.*

Received March 5, 1947

## INTRODUCTION

One of the earliest publications concerning the *in vitro* inactivation of estradiol by liver is that of Zondek (1) who showed that rat liver *brei*, when incubated with estradiol, brings about a loss of 95% of the estrogenic activity. Heller (2) found that rat liver slices also can inactivate estradiol. Both of these authors found that hydrolysis of the reaction mixture leads to only a very slight increase in its estrogenic activity. This indicates that esterification plays only a minor role in the loss of estrogenic potency.

With the exception of Heller's finding that the estradiol inactivation by liver is cyanide-sensitive, little experimental work has been done on the enzymatic nature of the process. A number of reports (3, 4, 5) suggest the participation of thiamine and riboflavin, but no direct evidence has been obtained.

This work was undertaken to secure some information on the mechanism of estradiol inactivation by rat liver. The results of high speed centrifugation of phosphate extracts of rat liver suggested that the cytochrome system might be involved. Further experiments provided evidence for the participation of another enzyme or enzymes in addition to cytochrome c and cytochrome oxidase.

<sup>1</sup> From a thesis submitted by Hilton Levy in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the Polytechnic Institute of Brooklyn, Brooklyn, N. Y.

<sup>2</sup> This work was supported by a grant from the Nutrition Foundation, New York, N. Y.

<sup>3</sup> Present address: Overly Biochemical Research Foundation, Inc., 254 W. 31st St., New York, N. Y.



## EXPERIMENTAL

## A. Materials and Methods

1. *Cytochrome c*. Cytochrome *c* was prepared from beef heart by the method of Keilin and Hartree (6). The concentration of the purified material in solution was determined spectrophotometrically, using the molar extinction coefficient,  $\beta$ , of  $6.48 \times 10^4 \text{ cm}^2 \text{ mole}^{-1}$  at 550 m $\mu$  for reduced cytochrome *c* as given by Theorell (7).

2. *Riboflavin*. Total riboflavin was determined by the method of Hodson and Norris (8).

3. *C $\gamma$  Alumina Gel*. *C $\gamma$*  alumina gel was prepared by the method of Willstätter and Kraut (9).

4. *Test for Enzymatic Inactivation of Estradiol*. 3 $\gamma$  of estradiol<sup>1</sup> were shaken with the material to be tested for enzyme activity for 2 hours at 37°C. at pH 7.4 in a total volume of 8 cc., unless otherwise stated. The enzyme action was then stopped by heating the reaction mixture in boiling water for 20 minutes. The contents of the flask were homogenized in a glass homogenizer (10) and assayed for the amount of residual estradiol by a modification of the uterine weight method of Lauson *et al.* (2). This method consists essentially of the injection into immature female rats of aliquots of the solution to be assayed. After 3 days the uterine weights are determined. Many preliminary standardization tests on aqueous solutions of pure estradiol showed that with any group of assay rats, the uterine weights were linearly proportional to the amount of estradiol given within the range 0.02–0.10 $\gamma$  of estradiol per rat. The slope of this line, however, varied somewhat from week to week. Accordingly, each week's group of assay rats were standardized by injecting five animals with 0.09 $\gamma$  each of pure estradiol, and five others with pure water. The average uterine weights found for these two control groups (*K<sub>i</sub>* and *K*) determined the slope and position of the uterine weight response line for that week. The mixtures to be assayed were diluted so that the maximum amount of estradiol each animal could receive would be 0.09 $\gamma$ . Only one dose level was used in each assay. From the standard line and the average uterine weight of the animals used in the assay of an unknown solution, the amount of estradiol in that solution was then obtained. At least 5 animals were used for each assay, and in some cases 10. Different preparations were not considered to have different enzymatic activities unless the amount of estradiol destroyed differed by more than 20%, except where sufficient assay animals were run to enable one to evaluate the results statistically.

Because of the possibility of the participation of thiamin or riboflavin as coenzymes in the reaction (3, 5), all enzyme assays described here were carried out in the presence of synthetic cocarboxylase (Merck) and of flavin adenine dinucleotide (F.A.D.). This latter was added in the form of either a heat-denatured, filtered kidney extract, a purified material kindly provided by Dr. David E. Green of Columbia University, or pure F.A.D. prepared by the method of Warburg and Christian (12).

These coenzymes alone, with cytochrome *c*, or with cytochrome *c* plus pure alumina gel had no effect on estradiol.

<sup>1</sup> The crystalline estradiol used was generously supplied by Dr. Erwin Schwenk of the Schering Corp., Bloomfield, New Jersey.

### *Preparation of Enzymatically Active Extracts*

5. *Phosphate Extract.* The procedure was a modification of the method used by Zondek (1) for the preparation of active, cell-free extracts of liver. Zondek extracted livers from infantile rats with *M/15*, pH 7.9 phosphate buffer. In the present experiments it was found, however, that livers from large Sprague Dawley rats of both sexes were as good a source of enzyme, if not better, than those of young rats, and that phosphate buffer at pH 7.4 gave slightly more active extracts. The pooled livers (fresh or stored in the frozen state) were ground with sand in three times their volume of buffer and extracted for two hours at 2°C. Tissue debris was sedimented at 3,000 r.p.m. for thirty minutes in the International 1 S.B. centrifuge, leaving a supernatant which will hereafter be referred to as *crude phosphate extract*. An extract prepared as described above from 3 g. of rat liver inactivated 70–100% of an added 3γ of estradiol in 2 hours at 37°C.

6. *Alumina Adsorbate.* The phosphate extract described above was stirred at 2°C. for one hour with Cγ alumina gel, 50 mg. dry weight of the gel being used/g. of original liver tissue. The gel was centrifuged off, washed rapidly with an equal volume of *M/15* phosphate buffer pH 7.4 to remove mechanically adhering liquid, and re-centrifuged. The packed, pink colored alumina adsorbate from 3 g. of liver varied somewhat in activity, but on the average it inactivated 60–80% of 3γ of estradiol in phosphate buffer at pH 7.4. Alumina gel alone did not adsorb or inactivate estradiol from such a solution.

7. *Phosphate Eluate.* It was found that treatment of the alumina adsorbate with water or acetate buffer at pH 5 led to only a little and often no elution of enzyme activity, while stirring the alumina adsorbate with 3 times its volume of *M/15* phosphate buffer, pH 7.4, at 2°C. for one hour gave an eluate containing 80–100% of the original enzyme activity.

### *B. Results*

1. *The Participation of Oxygen.* Anaerobic experiments were carried out in Thunberg tubes. In the side arm were placed 3γ of estradiol in 3 cc. *M/15* phosphate buffer, pH 7.4. The main tube contained 3 cc. of the crude phosphate extract of liver described above. The tubes were evacuated for 5 minutes before the contents were mixed. A control experiment was performed in the same way, except that, after evacuation, the tube was opened to the atmosphere. In the aerobic control the inactivation was complete, while under anaerobic conditions only 30–50% of the estrogenic activity disappeared.

2. *Evidence for the Participation of Cytochrome c.* If the cytochrome system functions as a terminal hydrogen acceptor from a "specific" dehydrogenating enzyme, a system containing estradiol and the "specific" enzyme should be capable of reducing cytochrome c. This reduction may be detected by the appearance of the reduced cytochrome absorption band at 550 mμ.

A crude "estradiol dehydrogenase" preparation was obtained as follows: the "phosphate eluate" described above was treated with saturated sodium dihydrogen phosphate solution until a pH of 5.0 was reached. The resulting cream colored precipitate was centrifuged off and suspended in *M*/15 phosphate buffer, pH 7.4, and used as the source of the enzyme. All preparations made in this way had only a slight cytochrome oxidase activity as shown by the Nadi color test.

Five preliminary experiments were performed. A Hollige hand spectroscope was used for visual observation and a Lumetron photoelectric colorimeter with 0.5 cm. deep cells was employed for the determination of relative transmission. These experiments were carried out in the presence of air, but since there was little agitation and the cytochrome oxidase activity of the preparations was low, there probably was not much cytochrome oxidase action. The details and results of these experiments are given in Table I. In every instance a band at 550  $m\mu$  appeared after 2-4 hours in the absorption cell containing the estradiol. Several hours later the band of reduced cytochrome c appeared in the control; but in every case, except one, the absorption at 550  $m\mu$  remained more intense in the estradiol cell up to 24 hours, at which time the experiments were terminated.

TABLE I

*Aerobic Reduction of Cytochrome c in Presence of Estradiol*

Control: Enzyme from 3 g. liver, 20 $\gamma$  cocarboxylase, 10 $\gamma$  F.A.D.,  $1 \times 10^{-6}$  moles oxidized cytochrome c. 0.01 cc. 95% ethanol. Total volume = 1 cc.

Experimental: Same as control, but instead of plain ethanol, 3 $\gamma$  estradiol in 0.01 cc. 95% ethanol. Room temperature.

*Per cent Transmission at 550  $m\mu$  (control cell set at 100)*

Exp.	After 2 hrs.	Estradiol cell After 24 hrs.
1	—	92
2	95	96
3	90	100
4	—	88
5	—	89

At a wave length of 640  $m\mu$  the transmission of the estradiol cell in Experiment 4 (against the control set at 100) was 99. This shows that changes in turbidity were not responsible for the differences in absorption at 550  $m\mu$ .

It will be seen from Table I that the tubes with estradiol contained more reduced cytochrome c than the controls. The variation in the results from one preparation to another might be explained by the presence of varying amounts of other reducing substances that competed with the estradiol for the ferricytochrome.

In another experiment, where  $7 \times 10^{-8}$  moles of oxidized cytochrome c were used per tube, the relative transmission at 550  $m\mu$  of the estradiol-containing cell after 40 hours was 52%. Animal assay of the

estradiol remaining in the solution showed that it had all been inactivated, presumably by oxidation.

Anaerobic experiments in Thunberg tubes gave somewhat more direct evidence of the participation of cytochrome c. The pH 5.0 precipitate of low cytochrome oxidase activity described above was used.

In the side arm of the control tube were placed  $7 \times 10^{-8}$  moles of oxidized cytochrome c, 20% of F.A.D., 20% of cocarboxylase, and 0.01 cc. of 95% ethanol. The main tube contained 0.3 cc. of enzyme suspension and 1.0 cc. of *M*/15 phosphate buffer at pH 7.4. The total volume was 2.8 cc. The experimental tube contained, in addition, 3% of estradiol in the 0.01 cc. of ethanol. Both tubes were evacuated for 5 minutes, then incubated at 37°C. for 5 hours. The *per cent* transmission was determined as described above, in cells 0.5 cm. deep. With the control set at 100, the transmission of the estradiol solution was 84 at 550  $m\mu$  and 98 at 640  $m\mu$ . Animal assay showed that complete inactivation of the estradiol had occurred. (Average uterine weight, 20 mg.;  $K = 20$  mg.,  $K_1 = 88$  mg.)

It should be pointed out that the difference in optical density between the two cells is not a quantitative indication of the amount of cytochrome c reduced by the oxidation of 3% of estradiol. In the first place, the control cell is actually not a proper blank for the estradiol-containing cell, since, at the end of the experiments, the control contains more oxidized cytochrome c, which has a marked absorption at 550  $m\mu$ . Secondly, the oxidation of other compounds may differ in the presence or absence of estradiol.

It will be noted that, whereas under anaerobic conditions, without excess cytochrome c, the inactivation of estradiol was previously found to be very incomplete, the addition of excess cytochrome c leads to complete inactivation. Thus under anaerobic conditions ferricytochrome c may presumably act as a hydrogen acceptor in the enzymatic dehydrogenation of estradiol.

3. *Role of Cytochrome Oxidase.* To obtain some idea of the particle size of the enzyme system, centrifugal studies were undertaken.

The first experiments were performed with the multi-speed attachment of the International Centrifuge, using crude phosphate extract. The extract was subjected to centrifugal fields varying from 1,800 *g* to 15,000 *g* for 30 minute periods. Enzyme activity was found in the sediment obtained at 3,600 *g*, in the supernatant at 15,000 *g*, and in every intermediate fraction. The supernatant from the 15,000 *g* centrifugation was still opalescent.

To attain higher centrifugal fields, the chilled quantity rotor of a Pickels ultracentrifuge was used. Phosphate extracts of rat heart and rat liver were subjected to a

force of 67,000 *g* (= 30,000 r.p.m.) for 30 minutes. Manometric cytochrome oxidase determinations were performed on the pellets and supernatants, with 5 mg. of *p*-phenylenediamine as substrate (13). Estradiol inactivating ability was also determined.

The results are given in Table II.

TABLE II  
*Correlation of Cytochrome Oxidase Activity and Estradiol  
Inactivating Activity*

Material assayed	Cytochrome oxidase activity, mm. <sup>3</sup> O <sub>2</sub> /hr./2.8 g. original tissue	Average uterine weight	Estradiol destroyed
		<i>mg.</i>	<i>Per cent</i>
Liver pellet <sup>1</sup> = 2.8 g. liver	800	26	80
Liver supernatant = 2.8 g. liver	4	53	22
Heart pellet <sup>1</sup> = 2.8 g. heart	960	32	70
Heart supernatant = 2 g. heart	108	67	0

<sup>1</sup> Pellet is material sedimentable at 67,000 *g* for 30 minutes.

The supernatant was completely clear. The sedimentation behavior of the enzyme complex shows qualitative similarity to that previously reported for cytochrome oxidase (14).

*The Effect of Inhibitors on the Enzymatic Activity of Crude Phosphate Extract of Rat Liver*

(a) It has been shown by Keilin and Hartree (6, 15) that cyanide and azide inhibit cytochrome oxidase activity. This inhibitory action is, however, not specific for cytochrome oxidase, but is also observed in the case of other heavy metal-containing enzymes.

A phosphate extract of liver was prepared in the usual way. A neutralized solution of the inhibitor was added to the extract in sufficient quantity to give a final concentration of  $1 \times 10^{-2}$  *M*. The mixture was allowed to stand for one hour at room temperature, after which 3γ of estradiol were added. The system was then incubated at 37°C. for 2 hours. Controls containing the extract alone were subjected to the same temperature changes, and estradiol was added at the same time as to the experimental vessels. Each flask had 3 cc. of extract, equivalent to 1 g. of original liver, plus 5 cc. of *M*/15 phosphate buffer, pH 7.4.

The results of a typical experiment are shown in Table III.

The cyanide inhibition as here observed is in agreement with the results of Heller (2). The data of Table III strongly suggest that we are dealing with heavy metal enzyme catalysis.

TABLE III  
*Inhibitory Action of Cyanide and Azide ( $10^{-2}$  M)*

Compound	Average uterine wt. mg.	Estradiol destroyed Per cent
Control	30	72
NaN <sub>3</sub>	45	38
KCN	50	26
K	18	
K <sub>2</sub>	61	

(b) *The Effect of Carbon Monoxide and Light.* It has been shown by Warburg and others (16, 17, 18, 19) that a mixture of 95% CO-5% O<sub>2</sub> will inhibit cytochrome oxidase activity in the dark, but that this inhibition is reversed by strong light, the amount of reversal being dependent on the wavelength of light used. Melnick (19) calculated, by extrapolation from results at adjacent wavelengths, that the most effective wavelength for the reversal of the carbon monoxide inhibition of cytochrome oxidase activity is 450 mμ. Since there exists no readily available source of monochromatic light at that wavelength, he used the 436 mμ line of the mercury spectrum.

The present experiments were conducted in cylindrical Warburg vessels of 50 cc. volume which had an inner, circular compartment of about 2.5 cm. diameter and an outer one of about 5 cm. diameter. In the inner compartment were placed 3 cc. of M/15 phosphate buffer, pH 7.4, plus 3γ of estradiol. The outer compartment contained 3 cc. of the crude phosphate extract of liver. The size of the flask was such that the two solutions, when mixed, formed a layer about 6-8 mm. deep. Carbon monoxide was generated by dropping hot concentrated sulfuric acid into formic acid. The gas was washed with sodium hydroxide and concentrated sulfuric acid, measured, and stored over water. To the carbon monoxide was added enough oxygen to give a concentration of the latter of 5% by volume. One vessel was painted black, the other was left clear. A General Electric H-3 mercury vapor lamp was fitted for under water use, and was covered by a glass filter transmitting radiation between 420-450 mμ. The only intense Hg line in this region is situated at 436 mμ. The lamp was placed obliquely under the transparent vessel, about 4 cm. from it.

The "light" and "dark" vessels were filled with the proper solutions, connected with the manometers, placed in the Warburg bath (at 37.5°C. except as noted), and gassed simultaneously with the CO-O<sub>2</sub> mixture. Forty-five minutes later the mercury lamp was turned on, and allowed to come to full intensity. After the extract had been incubated in this atmosphere for one hour, the estradiol-buffer solution was added and the mixture incubated for two additional hours. The usual assay procedure for remaining estradiol was then carried out. Ten animals were used in each assay, and the probable error <sup>5</sup> of the average uterine weight determined. From this the prob-

<sup>5</sup> Statistical analysis was carried out here because in some cases the difference between the amounts of estradiol inactivated by the enzyme-carbon monoxide in the light and in the dark was not as great as that previously set up as significant, i.e., 20%. The number of assay animals justified the use of statistical methods.

ability of the difference between the results being due to chance was calculated. The method described by Sherman (20) was used.

The results are summarized in Table IV.

TABLE IV  
*Effect of CO and Light on the Inactivation of Estradiol by Crude  
Phosphate Extract of One Gram of Rat Liver, at 37.5°C.*

Exp	Estradiol Destroyed per cent				
	1	2	3	4	5 <sup>2</sup>
Control	70	60	86	56	70
CO-O <sub>2</sub> dark	38	22	61	0	42
CO-O <sub>2</sub> light (436 mμ)	70	50	72	0	75
Probability <sup>1</sup>	7:1	20:1	4:1		400,000:1

<sup>1</sup> Probability against difference being due to chance.

<sup>2</sup> Experiment was performed at 25°C.

It may be concluded that carbon monoxide inhibits the inactivation of estradiol, and that light of wavelength 436 mμ reverses this inhibition.

## DISCUSSION

The experiments reported here demonstrate the participation of the cytochrome oxidase system in the inactivation of estradiol by extracts of rat liver. Whether the estradiol is converted to metabolites of lower estrogenic activity or of no estrogenic activity cannot be determined by the experimental methods used.

That the cytochrome oxidase system alone cannot inactivate estradiol is shown by several observations. In the first place, the active oxidant in the system, ferricytochrome c, is without effect on estradiol. Second, when a crude enzyme preparation is added to excess ferricytochrome c, however, loss of estrogenic activity results, even under anaerobic conditions. In the third place, almost all mammalian tissues contain cytochrome oxidase, but the ability to inactivate estradiol is restricted very largely to the liver. Indeed, the data presented in Table II show that the supernatant from the high speed centrifugation

of phosphate extract of rat heart retains significant cytochrome oxidase activity, but has no estradiol inactivating effect. Preliminary results suggest that the addition of pure F.A.D. to pH 5 acetate eluates of the alumina adsorbate leads to an increase in inactivating ability. These eluates by themselves have very little effect on estradiol. This augmentation of activity by F.A.D., however, is difficult to reproduce and requires further study.

These results suggest that a dehydrogenase as well as a terminal oxidation system is necessary for estradiol inactivation, but further experimentation is needed before this may be considered as established.

#### ACKNOWLEDGMENT

The author wishes to thank Professor Kurt G. Stern of the Polytechnic Institute of Brooklyn for his advice and guidance throughout this investigation.

#### SUMMARY

1. In the absence of oxygen the inactivation of estradiol by extracts of rat liver is markedly decreased.

2. Cytochrome c alone has no effect on estradiol. In the presence of liver extracts low in cytochrome oxidase activity, however, estradiol is inactivated and some of the added ferri-cytochrome is reduced.

3. Fractionation experiments in strong centrifugal fields indicate a particle size of the estradiol-inactivating enzyme complex of the order of that previously found for cytochrome oxidase.

4. The enzyme activity is inhibited by cyanide, azide and carbon monoxide. The carbon monoxide inhibition is reversed by intense light of wave length 436 m $\mu$ .

5. These results suggest that the inactivation of estradiol by rat liver extracts involves the cytochrome oxidase-cytochrome c system and presumably an additional enzyme, *e.g.*, a dehydrogenase.

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# Differential Inhibition of Respiration and Dark CO<sub>2</sub>-Fixation in *Scenedesmus* and *Chlorella*

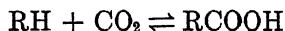
M. B. Allen,<sup>1</sup> H. Gest and M. D. Kamen

*From the Mallinckrodt Inst. of Radiology and Dept. of Chemistry,  
Washington Univ., St. Louis, Mo.*

Received March 10, 1947

## INTRODUCTION

In previous studies, using short-lived radioactive carbon (C<sup>11</sup>) as a tracer (1), it has been demonstrated that a variety of photosynthetic organisms fix carbon dioxide reversibly in the absence of light. Chemical evidence, while somewhat fragmentary, indicates that the major portion of the fixation in the alga, *Chlorella pyrenoidosa*, involves formation of carboxyl, according to the reaction:



in which RH is a chemical entity as yet unidentified. Supporting this conception are the many researches which in recent years have established the ubiquitous occurrence of carboxylation reactions in heterotrophic organisms (2, 3, 4). It is considered necessary at present to postulate that the dark fixation (carboxylation) reaction observed in experiments with C<sup>11</sup> is unique and distinct from carboxylation as observed in mammalian tissue, bacteria and other heterotrophes for the following reasons: (1) none of the usual intermediates (dicarboxylic or tricarboxylic acids) which have been demonstrated to be active in heterotrophic CO<sub>2</sub>-fixation are found to participate in dark CO<sub>2</sub>-fixation, (2) the molecular weights of the intermediates in the "photosynthetic" dark fixation reactions are much higher than is to be expected on the basis of any of the accepted mechanisms worked out for carboxylation in respiratory processes, and (3) the sensitivity of the dark fixation to various inhibitors (HCN, phenylurethane, ultraviolet

<sup>1</sup> National Research Fellow in Chemistry.

light, etc.) parallels the overall inhibition of photosynthetic oxygen evolution and not respiratory oxygen uptake. Thus, in *Chlorella pyrenoidosa* (1), it has been found that  $10^{-2}$  M KCN depresses both dark  $\text{CO}_2$ -fixation and overall oxygen evolution to less than 0.3% of the normal rate, while the rate of endogenous respiration is practically unaltered. Even more remarkable effects are observed using the inhibition by irradiation with the 2537 Å Hg line. In this case, photosynthetic oxygen evolution and dark  $\text{CO}_2$ -fixation are both diminished quantitatively in the same ratios (5% of normal) while no change is observed in respiration. In the absence of chemical elaboration of the intermediates involved, which is, of course, the only direct proof of participation of dark  $\text{CO}_2$ -fixation in the photosynthetic mechanism, this type of evidence remains the basis for the present concept of a carboxylation reaction as a primary step in photosynthetic fixation of carbon dioxide.

Gaffron (5) has shown that it is possible with the alga *Scenedesmus*<sup>2</sup> to inhibit endogenous respiration almost completely without affecting photosynthesis markedly. There is thus afforded the opportunity to observe the course of dark fixation relative to overall photosynthesis under conditions opposite to those obtaining in *Chlorella*. Hence, it is of interest to extend the previous observations on *Chlorella* to *Scenedesmus* by use of the same methods involving tracer carbon. In this report there will be presented and discussed results obtained in such studies.

## EXPERIMENTAL

### *Culture of Algae*

*Scenedesmus* of Gaffron's D<sub>3</sub> strain were grown in the bicarbonate culture solution described by him (5). A 5%  $\text{CO}_2$ -95% air gas mixture was bubbled through the culture flasks, which were continuously illuminated by a bank of fluorescent lights. *Chlorella pyrenoidosa* were grown in the same manner. Adequate growth was obtained in 4 days, and algae were regularly harvested at the end of this interval, so that all cultures used were of the same age. For some experiments, the algae were "starved" in the following manner. The algae were centrifuged from the culture solution and suspended in 0.1 M  $\text{NaHCO}_3$  solution. This suspension was then allowed to stand in the dark for 24 hours before using.

<sup>2</sup> We are indebted to Prof. Gaffron for the *Scenedesmus* strain used in these studies.

*Manometric Measurements*

Algae, 20–25 mm.<sup>3</sup>, were suspended in 5 cc. of 0.1 *M* NaHCO<sub>3</sub> or in Warburg's No. 9 buffer and measurements of oxygen exchange made in the usual Warburg apparatus. For photosynthesis measurements, illumination (saturation intensity) was provided by a bank of Mazda bulbs above the bath. For respiratory readings the apparatus was covered with a dark cloth. Temperature was maintained at 23°C.

*Radioactive Measurements*

For measurement of CO<sub>2</sub> uptake in the dark, ca. 100 mm.<sup>3</sup> algae were washed, suspended in 3 ml. water, and placed in a Thunberg tube, which was wrapped in a dark cloth.

Labeled 0.1 *M* NaHCO<sub>3</sub> solution was prepared in the following manner. A small quantity of BaC<sup>14</sup>O<sub>3</sub> received from the Manhattan Project Isotope Research Division was weighed accurately and mixed with sufficient inactive BaCO<sub>3</sub> so that, in the resultant operations, sufficient carbonate could be obtained to make approximately 25 cc. of 0.1 *M* NaHCO<sub>3</sub> solution. The BaCO<sub>3</sub> was placed in one arm of an apparatus equipped with a pump out and two side arms (see Fig. 1). In one side arm, a small vial containing perchloric acid was placed. The other arm contained sufficient 1 *M* NaOH (CO<sub>2</sub>-free) to absorb the quantity of C<sup>14</sup>O<sub>2</sub> liberated when the acid was tipped into the carbonate. Before tipping, the apparatus was evacuated. To prevent bumping of the alkali solution during evacuation, the alkali was frozen by immersion in liquid air. The alkali was allowed to warm gradually to room temperature, the residual gases thus being liberated in a controllable fashion. In some instances, the freezing and thawing cycle was carried through twice, before the final evacuation. With the alkali solution thawed under a vacuum of 1 mm. Hg, the acid was tipped into the solid carbonate cautiously and the C<sup>14</sup>O<sub>2</sub> volatilized into the alkali solution. An indicator such as phenol red was used to ascertain that the pH of the absorbing solution remained on the alkaline side. After all evidence of reaction had ceased, the acid end of the apparatus was warmed gently to drive over residual C<sup>14</sup>O<sub>2</sub>. The alkaline solution now consisted of 1 *M* NaHCO<sub>3</sub> which was ready for dilution and use. The radioactivity assayed from 10<sup>4</sup>–10<sup>6</sup> ct./min./cc. after final dilution. In the researches discussed only bicarbonate solutions were employed so

that the manometric and radioactive measurements could be considered strictly comparable.

Two cc. labeled  $0.1\text{ }M\text{ NaHCO}_3$  were introduced by pipette into the Thunberg tube containing the algal suspension which was then incubated in a dark room for 40–60 minutes. After this period, the tube was connected to two traps in series which were immersed in liquid air. The first trap was empty, while the second contained 2–3 cc.  $1\text{ }N\text{ NaOH}$ . One cc.  $6\text{ }N\text{ HCl}$  was tipped into the Thunberg tube

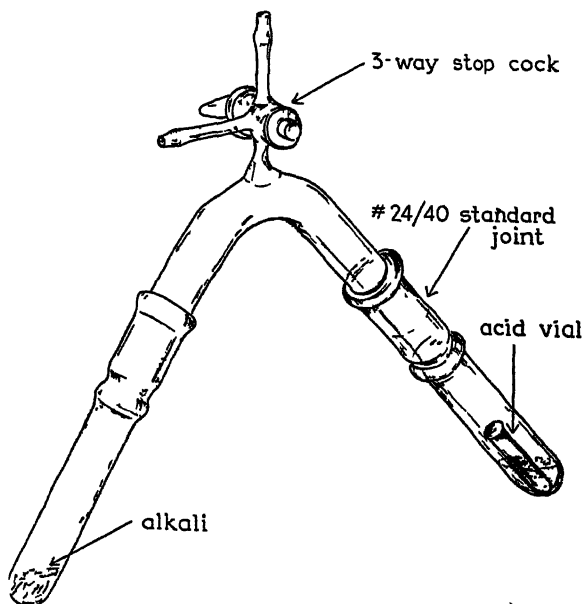


FIG. 1. Apparatus for preparation of labeled  $\text{NaHCO}_3$  solutions.

from its cap, the tube placed in a boiling water bath and a vacuum pumped on it through the traps. Substantially all the  $\text{CO}_2$  was removed from the algal suspensions and recovered in the traps after 5 minutes of this treatment. A small amount of inactive  $\text{NaHCO}_3$  was then added to the acid solution to dilute any traces of radioactive bicarbonate which might remain, after which the solution was evaporated to dryness on a steam bath. A blank experiment showed that all active  $\text{CO}_2$  was removed by this treatment. In a control experiment, in which

part of the sample was evaporated in a vacuum desiccator and the remainder on the steam bath, it was shown that no activity was lost by hot evaporation.

Few experiments with C<sup>14</sup> have been reported previously (6, 7, 8) so that a description of the method of measurement used is warranted. Since the material assayed in these experiments was thermostable, solid, and not deliquescent, and since it was not essential to determine the position of the fixed carbon in a particular molecule it was not necessary to convert the samples to BaCO<sub>3</sub>. The residue from the above evaporation was taken up in a few ml. of water and an aliquot evaporated on a thin aluminum disc, using an infrared lamp above the sample. This was found to be a quick and convenient way of preparing uniform reproducible samples. The aluminum disc was weighed before and after evaporation of the sample on it, and the self-absorption correction calculated from this weight and the area covered by the sample (a curve for calculating self-absorption correction has been published by Reid (9)). Most of the samples used were sufficiently thin (<1 mg./cm.<sup>2</sup>) so that the absorption correction was less than 5%.

The aluminum disc was placed in a sample holder which brought it into a reproducible position close to the counter window and assayed with a bell-jar type counter having a thin mica window (10). The counter was filled with a mixture of 9 cm. argon and 1 cm. ethanol and connected to a scale of 64 circuit and mechanical recorder. Since the geometry was constant, it was not necessary to make corrections other than those for self-absorption. Thus, C<sup>14</sup> samples were measured with little more difficulty than those of isotopes with harder radiations. Examples of the precision obtainable, even with weak samples, are shown in Table I.

TABLE I

*Reproducibility of C<sup>14</sup> Measurements*

Sample	Activity c./min.
Sample No. 1	130 ± 5
Duplicate sample No. 1	136 ± 5
Sample No. 1 evaporated hot	130 ± 5
Sample No. 2	193 ± 6
Duplicate sample No. 2	206 ± 6
1 cc. soln. No. 3	48 ± 3
2 cc. soln. No. 3	98 ± 3

## RESULTS

Inhibition of photosynthesis and respiration (measured manometrically) of *Scenedesmus* by KCN is shown in Fig. 2. Typical data on the relative inhibition of photosynthesis, respiration, and dark  $\text{CO}_2$  fixation in *Scenedesmus* are given in Table II, while similar data for *Chlorella* appear in Table III. It will be noted that in starved *Scenedesmus* cells (Expts. III and IV, Table II), it was possible to obtain

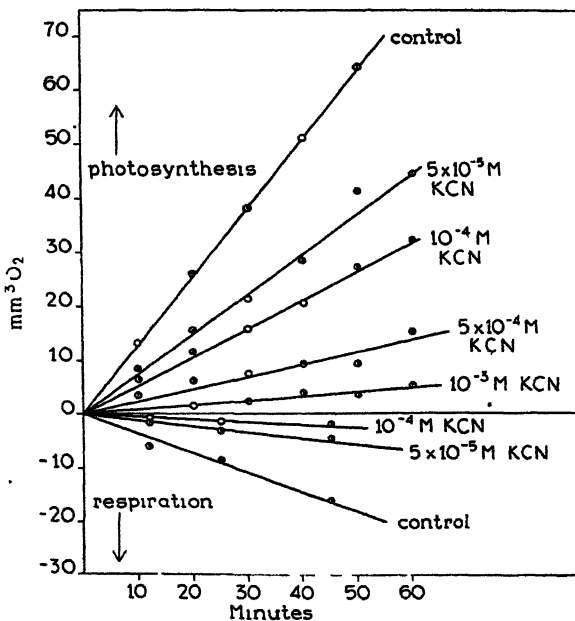


FIG. 2. Manometric measurements; cyanide inhibition of photosynthesis and respiration of *Scenedesmus*.

enhanced differential inhibition of photosynthesis and respiration as compared with unstarved cells (Expts. I and II, Table II). It should be emphasized that since only relative fixation values were of interest, it was unnecessary to standardize the  $\text{C}^{14}$  dosage from run to run. Hence the  $\text{C}^{14}$  fixation exhibited by the controls in these tables are not comparable.

TABLE II  
*Differential Inhibition of Photosynthesis, Respiration, and  
 CO<sub>2</sub> Fixation in Scenedesmus*

Unstarved cells (freshly harvested)						
	I	II	III	IV	V	VI
Exp. I	Photosyn- thesis mm. <sup>3</sup> O <sub>2</sub> /min./mm. <sup>3</sup> cells	Respiration mm. <sup>3</sup> O <sub>2</sub> /min./ mm. <sup>3</sup> cells	CO <sub>2</sub> fixation c./min./mm. <sup>3</sup> cells <sup>1</sup>	Rel. p.s.	Rel. resp.	Rel. CO <sub>2</sub> fixation
Control	0.360	-0.034	18.0	1.00	1.00	1.00
10 <sup>-4</sup> M KCN	0.241	-0.009	12.8	0.67	0.26	0.55
5×10 <sup>-4</sup> M KCN	0.079	0.000	9.9	0.24	0.00	0.47
Exp. II						
Control	0.173	-0.040	27.4	1.00	1.00	1.00
10 <sup>-4</sup> M KCN	0.070	0.000	—	0.40	0.00	—
5×10 <sup>-4</sup> M KCN	0.028	0.000	9.6	0.16	0.00	0.35
Starved cells (24 hours in dark in NaHCO <sub>3</sub> before using)						
Exp. III						
Control	0.273	-0.080	15.5	1.00	1.00	1.00
5×10 <sup>-4</sup> M KCN	0.233	-0.002	8.6	0.85	0.02	0.46
Exp. IV						
Control	0.141	-0.019	16.9	1.00	1.00	1.00
10 <sup>-4</sup> M KCN	0.083	-0.007	—	0.59	0.37	—
5×10 <sup>-4</sup> M KCN	0.062	0.000	27.1	0.43	0.00	1.60

<sup>1</sup> Different activities of NaH<sup>14</sup>C<sub>3</sub>O<sub>3</sub> were used in different sets of experiments, so fixation in one set cannot be compared with that in another.

Since the results given in these tables suggested that a portion of the CO<sub>2</sub> fixation in these algae might occur *via* a respiratory mechanism, a search was made for activity in respiratory products which have been found to fix CO<sub>2</sub> in other organisms. A carrier mixture of organic acids containing citric, fumaric, acetic, butyric, pyruvic, and α-ketoglutaric acids was added to the algal extract.<sup>3</sup> Fumaric acid was separated from

<sup>3</sup> The presence in these algae of α-ketoacids (pyruvic, α-ketoglutaric) to the extent of 0.01% by weight has been demonstrated (unpublished experiments).



TABLE III  
*Differential Inhibition of Photosynthesis, Respiration, and  
 CO<sub>2</sub> Fixation in Chlorella*

Unstarved cells (freshly harvested)

Exp I	Photosynthesis mm <sup>3</sup> O <sub>2</sub> /min / mm <sup>3</sup> cells	Respiration mm <sup>3</sup> O <sub>2</sub> /min / mm <sup>3</sup> cells	CO <sub>2</sub> fixation c/min/mm <sup>3</sup> cells <sup>1</sup>	Rel p s	Rel resp	Rel CO <sub>2</sub> fixation
Control	0.231	-0.009	5.00	1.00	1.00	1.00
10 <sup>-2</sup> M KCN	0.013	-0.013	2.80	0.00	1.45	0.56
Exp II						
Control	0.125	-0.006	33.4	1.00	1.00	1.00
10 <sup>-2</sup> M KCN	0.013	-0.008	6.50	0.00	1.33	0.19

Starved cells (24 hours in dark in Warburg No. 9 buffer before using)

Exp III						
Control	0.296	-0.014	4.00	1.00	1.00	1.00
10 <sup>-2</sup> M KCN	0.017	-0.017	4.40	0.00	1.19	1.10
Exp IV						
Control	0.185	-0.009	17.1	1.00	1.00	1.00
10 <sup>-2</sup> M KCN	0.006	-0.006	11.0	0.00	0.73	0.65

<sup>1</sup> Different activities of NaHC<sup>14</sup>O<sub>3</sub> were used in different sets of experiments so fixation in one set is not comparable with that in another.

this mixture by precipitation of mercurous fumarate in dilute nitric acid solution. The precipitate was dissolved, reprecipitated twice, and counted. Results are shown in Table IV. The keto acids were converted to the 2,4-dinitrophenylhydrazones, which were purified by extracting with ether, extracting from the ether with 10% Na<sub>2</sub>CO<sub>3</sub>, acidifying, and reextracting with ether. Small amounts of activity were found in the keto acid fraction, but an accurate estimation of this activity could not be made because of the tendency of the hydrazones to decompose and react with the aluminum sample discs.

The residues after water treatment invariably contained less than 10% of the total C<sup>14</sup> fixed. This water-insoluble radioactive material,

while only a small fraction of the total radioactivity, was lumped with the supernatant water-soluble material in the determination of the dark CO<sub>2</sub>-fixation.

TABLE IV  
*Activity of Fumarate Fractions in Chlorella and Scenedesmus CO<sub>2</sub>-Fixation<sup>1</sup>*

<i>Scenedesmus</i> Samples	Total activity	Fumarate activity
Unstarved control	3735 ± 70	36 ± 2
Unstarved + KCN (5 × 10 <sup>-1</sup> M)	2900 ± 60	22 ± 2
Starved control	3860 ± 70	72 ± 2
Starved + KCN (5 × 10 <sup>-1</sup> M)	2570 ± 70	24 ± 2
<i>Chlorella</i> Samples		
Unstarved control	5012 ± 80	130 ± 5
Unstarved + KCN (10 <sup>-2</sup> M)	972 ± 40	
Starved control	5126 ± 80	118 ± 5
Starved + KCN (10 <sup>-2</sup> M)	3316 ± 70	226 ± 6

<sup>1</sup> Errors shown are the standard deviations calculated from counting data.

## DISCUSSION

For greater clarity, the results obtained with each organism will be considered separately. In *Scenedesmus*, the first feature of the data which may be noted is that the course of CO<sub>2</sub>-fixation in the dark parallels photosynthetic activity and not endogenous respiration (Table II, Cols. IV and VI). In fresh cells, this parallelism is exhibited in a regular fashion, whereas in cells with depleted reserves little correspondence is found.

General experience indicates that the relative extent of the fixation reactions in the presence of cyanide is markedly influenced by the previous history of the cells. This state of affairs is to be expected because in the reaction chains leading to CO<sub>2</sub>-fixation, whether in the respiratory cycle or the photosynthetic metabolism, differential sensitivity to cyanide of component reactions will determine the contribution of respiratory fixations to the total fixation observed. The significant results are those obtained when cyanide concentrations are employed in which respiratory activity is abolished while photosyn-

thesis is still proceeding at a rate comparable with that shown in cells not exposed to cyanide. In fresh cells it is seen that under conditions where respiration is lowered to less than 2% of the normal rate (essentially zero activity) and photosynthesis is lowered to about 20% of normal, dark fixation is observed to remain at about 40% of normal. In starved cells, the data are more irregular but present substantially the same picture. In one experiment (Expt. IV) the cells actually showed a considerable increase (60%) above normal although photosynthetic activity was reduced by 50%. However, in other experiments, of which Expt. III, Table I, is typical, the level of CO<sub>2</sub>-fixation was lowered more than that of the overall photosynthetic activity.

No ready explanation for the irregularities introduced by depletion of reserve material in *Scenedesmus* suggests itself. An attempt to correlate this behavior with induction of respiratory CO<sub>2</sub>-fixation reactions by an effect such as mobilization or labilization of endogenous reserves was made, using as an index of respiratory CO<sub>2</sub>-fixation the appearance of labeled carbon in fumaric acid. In Table IV, results of such an experiment indicate a significant rise in fumarate C<sup>14</sup> level in the starved control. In the presence of cyanide, this fumarate-fixed carbon dropped to the same level as in the unstarved cyanide treated cells. Such results are in good accord with those to be expected on the basis of increased respiratory fixation. The results of Expt. IV are not explicable on this basis. However, it should be noted that in Expt. IV, the cells were considerably less active both in respiration and photosynthesis than cells such as those used in Expt. III. It is also conceivable that under the proper culture conditions differential inhibition of overall photosynthesis favoring accumulation of thermal fixation products can occur, a condition thus obtaining which would lead to a result such as that observed in Expt. IV.

The relative magnitude of the CO<sub>2</sub>-fixation may be defined as the level of C<sup>14</sup> fixed in 45 minutes in the dark compared with the fixation occurring during photosynthesis for the same time. The relative magnitude of dark fixation is found to be 2-5%, a result in agreement with the results described in the early studies with C<sup>14</sup>. The contribution of dark fixation reactions other than those associated with thermal photosynthetic processes would appear to be small as judged from the activities observed in the fumarate fractions as well as in the  $\alpha$ -keto

acid fractions. A maximum estimate would place such radioactivity at <10% of the total fixation observed in normal unstarved cells.<sup>4</sup>

In this connection it may be remarked that the magnitude of the CO<sub>2</sub>-fixation observed is far greater than would be expected from the respiratory activity of the algae. Previous experience indicates that in normal heterotrophic CO<sub>2</sub>-fixation (*i.e.*, in yeast) the amount of CO<sub>2</sub> evolved exceeds the amount of labeled CO<sub>2</sub> fixed by a factor of 5-10. In these experiments CO<sub>2</sub>-fixation is observed to be nearly equal to respiratory CO<sub>2</sub> evolution. This would imply a factor of only 1 as against the usual 5 or 10. It seems reasonable to conclude on this basis that only a small fraction of the dark fixation observed can be due to respiratory activity.

It appears from these observations that in *Scenedesmus* dark fixation of CO<sub>2</sub> as well as overall oxygen evolution can be dissociated from endogenous respiration by the use of cyanide. Manometric experiments show that oxidation of external substrates such as glucose is inhibited in the same way as oxidation of endogenous reserves, so that it is reasonable to assume that similar dissociation of CO<sub>2</sub> fixation from CO<sub>2</sub> fixation reactions induced by glucose oxidation can occur. This point has not been tested directly.

Turning to the results obtained with *Chlorella*, it is seen that no such unambiguous conclusions can be drawn regarding the relative course of dark CO<sub>2</sub>-fixation, photosynthesis, and endogenous respiration. In normal unstarved cells the dark fixation appears to parallel roughly the response to cyanide observed for photosynthesis. Thus in Expts. I and II, Table III, it may be noted that while the photosynthetic rate is cut practically to zero, the dark CO<sub>2</sub>-fixation also shows a drop but only by a factor of 2-5. However, respiration under the same conditions rises from 30% to 50%. From this rough parallelism between dark CO<sub>2</sub>-fixation and overall photosynthesis it is evident that, in both *Chlorella* and *Scenedesmus*, much the same conclusions can be drawn concerning the dependence of dark CO<sub>2</sub>-fixation on the photosynthetic mechanism.

<sup>4</sup> In arriving at this estimate, it has been assumed that the specific C<sup>14</sup> content of the carboxyl groups in fumaric acid is representative of all carboxyl groups in the carboxylic acid intermediates which may be involved and that the number of such intermediates is given by the usual reaction chain postulated for the tricarboxylic acid cycle.

There appears to be a much closer coupling between photosynthesis and respiration in *Chlorella* than in *Scenedesmus*. The overall photosynthetic activity of both organisms shows approximately the same response to cyanide over the range of concentrations from  $10^{-3}$  to  $10^{-5}$  *M*. However, while respiration in *Scenedesmus* is completely inhibited at  $5 \times 10^{-4}$  *M* cyanide, respiration in *Chlorella* is untouched and in fact enhanced at concentrations of cyanide two orders of magnitude greater. This drastic difference in respiratory sensitivity of the two organisms makes it possible to find conditions whereby it is possible to observe differential inhibition of respiration in favor of photosynthesis in *Scenedesmus*.

It has not been possible to achieve in our strain of *Chlorella* as clear cut a quantitative parallelism between cyanide inhibition of dark  $\text{CO}_2$ -fixation and photosynthesis as reported previously (1). However, the strains used may not be strictly identical with those used in the previous studies, some six years and an untold number of transfers having occurred in the interim.

A basis for an understanding of the source of discrepancies found in the "same" organism can be inferred from the observations on *Chlorella* treated so that endogenous stores are depleted. In Table III, Expts. III and IV, it is seen that dark fixation parallels the endogenous respiration rather than photosynthesis. All  $\text{CO}_2$ -fixation observed under these conditions can be ascribed to respiratory processes. Such a conclusion is borne out by the fumarate  $\text{C}^{14}$  level observed (Table IV) in the cyanide-treated starved *Chlorella* cells, in which the relative fixation in fumarate is more than 10 times as great as in the corresponding case for *Scenedesmus*.

The significance of these observations for future researches on the isolation of photosynthetic intermediates is obvious. It would appear that *Chlorella* is a most unreliable organism to use as a source for the dark fixation products produced in the photosynthetic process since, by variation of the culture conditions, one may abolish practically all dark pickup which may be ascribed to the photosynthetic mechanism. On the other hand, it appears that in *Scenedesmus* it should be possible by incubation of mass cultures in the presence of cyanide ( $2-5 \times 10^{-4}$  *M*) to produce organisms containing maximal quantities of dark  $\text{CO}_2$ -fixation products contaminated with a minimum of substances formed from non-photosynthetic fixation reactions. .

## SUMMARY

1. The magnitude of dark CO<sub>2</sub>-fixation reactions in the presence of cyanide has been studied in two species of photosynthetic algae, *Chlorella pyrenoidosa* and *Scenedesmus* (Gaffron's D-3). As others have shown, it has been found possible by suitable variation of the cyanide concentration to establish conditions under which endogenous respiration and photosynthesis are inhibited differentially, the former process being maintained in *Chlorella*, the latter in *Scenedesmus*.

2. With normal healthy cells it is possible to demonstrate in both algal types a parallelism between the dark CO<sub>2</sub>-fixation reactions and overall photosynthetic activity. No relation is found to exist between endogenous respiratory activity and dark CO<sub>2</sub>-fixation. Under these conditions, CO<sub>2</sub>-fixation appears due mainly to dark reactions involved in the photosynthetic mechanism.

3. By "starving" the algae, it is possible to alter, and, in the case of *Chlorella*, to abolish completely, the parallelism between dark CO<sub>2</sub>-fixation and the ability to carry on normal photosynthesis. Under these conditions it appears that the major fraction of CO<sub>2</sub>-fixation reactions is owing to heterotrophic respiratory fixation reactions. This conclusion is deduced from the increased fixation in conventional intermediates, *i.e.*, dicarboxylic acids (of which fumarate is taken as an example) and  $\alpha$ -keto acids.

4. The importance of these results for the elaboration of isolation procedures for dark CO<sub>2</sub>-fixation products due to the photosynthetic mechanism is discussed.

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# The Significance of Impurities on the Biochemical Effects of Streptomycin

Robert H. Silber, Curt C. Porter,  
Martin Winbury and Irwin Clark

*From the Merck Institute for Therapeutic Research, Rahway, N. J*

Received March 13, 1947

## INTRODUCTION

Since the discovery of streptomycin by Schatz *et al.* in 1944 (1), relatively little information concerning the biochemical effects of this antibiotic in animals has been reported. Since the streptomycin preparations clinically available vary in purity from approximately 300 to 600 units or  $\gamma$  of streptomycin base per mg. solids (about 36–72% pure), a study of the effects of such material seemed indicated. Some biochemical effects of the administration of streptomycin of varying purity to dogs, monkeys, and rats have been reported by one of us in connection with a pharmacological study of streptomycin in animals (2). Among the toxic effects and biochemical changes noted in these animals were loss of appetite, decrease in body weight, proteinuria, slight increases in blood urea and fat, bromsulfalein retention, fatty changes in the liver and kidney, disturbance in equilibrium, edema, and a histamine-like effect on blood pressure. The conclusion that these effects were caused by the antibiotic principle could not be drawn because of the presence of undefined impurities. The lack of uniformity in the effects observed—in particular, the blood pressure effect and the intravenous toxicity—strongly suggested that at least some of them were due to the impurities rather than to the active principle. The purpose of this study was to further investigate the biochemical changes following the administration of streptomycin of varying purity in order to better evaluate the toxicity of streptomycin itself.

## METHODS

The methods employed were as follows: Serum protein, Lowry and Hunter (3); Albumin-globulin ratio in serum, Howe (4); Albumin-globulin ratio in urine, Hiller's



method, (5); Plasma fibrinogen, Cullen and Van Slyke (6); Urea nitrogen, Conway and O'Malley (7); Alkaline serum phosphatase, Bodansky (8); Cholesterol, free and total, Sheftel (9); Blood sugar, Horvath and Knehr (10); Creatinine and creatine, Peters (11); Blood fat and liver lipides, Bloor (12); Serum chloride, Shales and Shales (13); Urinary protein, Shevky and Stafford (14); Serum thymol turbidity, Shank and Hoagland (15). In the bromsulfalein retention test (Rosenthal and White, 16), 5 mg. of dye/kg. were injected in 1 minute and blood samples were taken after 5 and 15 minutes for dogs, and after 15 and 30 minutes for monkeys. Urinary reducing substances were determined as sugar except that tungstic acid precipitation was omitted. When proteinuria developed in animals given streptomycin, the Shevky-Stafford method was checked by trichloroacetic acid precipitation.

*Diuresis and Concentration Test.* On the day prior to the test 300 ml. of water/kg. of dog were administered by stomach tube at 5:00 P.M. and food was removed from the cage. Urine was collected without catheterization at 9:00 A.M. the following morning. The water pan was then removed from the cage and 300 ml. of water were administered by stomach tube. At 11:00 A.M. the third and last dose of water was administered. The bladder was emptied by catheterization 0, 4, 8 and 24 hours later.

*Phenolsulfonphthalein Excretion.* At 11:00 A.M. during the diuresis test, a solution of 1 or 2 mg. of dye was injected subcutaneously and the first and second 4-hour urines were assayed in a photoelectric colorimeter for dye content. A blank of the 9-11 A.M. urine was included to correct for urine pigment.

*Urea Clearance.* The urea excreted during the first 4 hours of the diuresis test was determined and the blood concentration was measured at the middle of the collection period for calculation of the ml. of blood cleared of urea per minute. Urea was not administered.

## EXPERIMENTAL RESULTS

With early lots of streptomycin, the flushing, nausea and drop in blood pressure were associated with the presence of a histamine-like impurity. When such lots, which showed histamine-like action on the blood pressure in cats, were incubated at pH 7.2 and 37°C. with histaminase,<sup>1</sup> the blood pressure effect was eliminated without change in antibiotic activity. Although the impurity was not isolated, some information regarding its identity was obtained. Like histamine, this factor could be extracted from alkaline solution with isoamyl alcohol. The blood pressure effect of such extracts paralleled the intensity of the color developed in the Pauly test for imidazole as well as the amount of ammonia released by incubation with histaminase. Furthermore, the absorption spectrum of the extracted material coupled with diazotized sulfanilic acid, differed from that obtained with histamine

<sup>1</sup> Prepared essentially by the method of Laskowski, M., Lemley, J. M., and Keith, C. K. *Arch. Biochem.* 6, 105 (1945).

only in exhibiting a small subsidiary absorption maximum attributable to other impurities.<sup>2</sup> Like histamine, the factor coupled in alkaline solution with *p*-(*tert*-amyl)-benzenediazonium chloride to form an insoluble precipitate.

The toxicity of streptomycin is also influenced by an impurity or impurities other than the histamine-like factor. This is indicated by the finding that there was great variation in the intravenous L.D. 50 for mice when samples of high potency, free of the histamine-factor, were injected (2). Representative data of other effects of impure streptomycin are summarized in Table I.

The proteinuria of one dog decreased to 5 g./l. 2 weeks after the streptomycin treatment had been discontinued. The other dog, which had a concentration of 46 g. of protein/l. of urine after having received injections for 3 weeks, showed a complete regression of the proteinuria within 4 weeks, in spite of continued administration of streptomycin. Since these dogs were treated with streptomycin from several different lots, it is possible that the regression of proteinuria may have resulted from an inadvertent change to a less toxic lot.

Other changes, such as the decrease in serum protein and the increases in plasma creatine and blood lipide were apparently related to a decrease in food intake and loss of body weight. Again, a change in the lot of drug administered may have been responsible for the improvement in appetite and body weight which occurred in spite of the continued injections.

The depression of urine volume was observed only during the period of streptomycin treatment. Immediately after discontinuation of the drug, the urine volumes increased, indicating that the depression was due to a circulatory or vasomotor change rather than to kidney damage. This was borne out by diuresis-concentration tests which revealed a depression immediately after the administration of streptomycin, but a relatively normal diuresis 1 or 2 days after the last injection. The inhibitory effect of impure streptomycin on water diuresis was partially eliminated by histaminase treatment. The slight increases in blood urea were probably related to this effect on the kidney.

Mushett and Martland (17) found histological evidence of fatty livers in monkeys injected with streptomycin (25 mg./kg./day intra-

<sup>2</sup> We are indebted to Dr. C. W. Rosenblum of the Merck Research Laboratories for determining the absorption spectra of these solutions.

TABLE I  
*Effects of Impure Streptomycin in Animals*

Potency γ/mg. solids	Species	Dose	Effects
250-310	Dog	50 mg./kg./day for 10-13 weeks S.C. or 100 mg./kg./day for 20 days, in 3 portions daily.	Proteinuria, up to 46 g./l. with A/G ratio usually under 1.0. Serum protein decrease from 5.5% to 3.9% with a decrease of 50% in A/G ratio. Blood lipid increase, 50-65%. Plasma fibrinogen increase of 250-700 mg.-%. Plasma creatine increase 2-3-fold. Decrease of 40% in body weight.
140-400	Monkey	25 mg. or 200 mg./kg./day for 5 days S.C. in 3 portions daily.	Bromsulfalein retention, 5-10% after 30 min. Blood urea nitrogen increase from 11 mg.-% to 23 mg.-%. Serum protein decrease. 7.0 g.-% to 6.0 g.-%.
300-600	Monkey	100 or 200 mg./kg./day for 5 days S.C. or I.V. in 3 portions daily.	Urine volumes depressed 50% during injection period. Proteinuria, up to 4.5 g./l.
30	Monkey	100 mg./kg./day for 5 days S.C. in 3 portions daily.	Edema in abdominal region by fourth day. Serum protein decrease from 7.2 g.-% to 4.7 g.-%. Mild proteinuria, up to 2.2 g./l. after termination of injections.

venously for 5 days). Analyses of 5 of these livers showed that the concentration of total lipid was about 20% higher than in 4 untreated monkeys. There was no increase in cholesterol concentration.

The rat appeared to be more resistant to the effects of impure streptomycin than the dog or the monkey. Ten adult rats which received 100 mg./kg. subcutaneously, daily, for 2.5 months not only survived, but showed no changes in urinary analyses, blood urea nitrogen, or plasma fibrinogen. This species difference was also borne out by the absence of pathological changes in these rats (17). Similarly,

after single subcutaneous injections of 100–200 mg./kg. to adult rats, no significant changes were found.

Since the preceding experiments, conducted with more or less impure streptomycin, showed that daily injections of 50–200 mg./kg. had occasional effects in dogs and monkeys, particularly on the kidney and liver, an experiment with more highly purified streptomycin was indicated. Because chemically pure streptomycin was not available for these experiments two highly purified lots were employed.<sup>3</sup>

Lot No. 143 had a potency of 725  $\gamma$ /mg. of solids, an intravenous L.D. 50 of 2670 units/20 g. mouse, and had no histamine-like activity. Lot No. 404 contained 640  $\gamma$  of streptomycin base/mg., had an intravenous L.D. 50 of 2000 units/20 g. mouse, and was also free of the histamine-like impurity. On the basis that pure streptomycin hydrochloride has a potency of 840 units/mg., these samples were about 86% and 76% pure, respectively.

Two adult male Beagle hounds (No. 769 and No. 829), weighing about 10 kg. each, were injected subcutaneously with 100 mg. of streptomycin/kg. daily, in 3 divided portions, at 9:00 A.M., 1:00 P.M., and 5:00 P.M., for a total of 20 days. They were fed Purina dog food *ad libitum* plus a daily supplement of 100 g. of horse meat. Two other Beagle hounds served as paired feeding controls with adjustment made for differences in initial body weights. Urines were collected under toluene between 4:00 P.M. and 10:00 A.M., at least 4 times weekly, with food and water removed from the cage. Blood samples were taken weekly after overnight fasting.

No significant changes in body weight or in food consumption resulted from the injection of streptomycin. The results of urinary analyses are recorded in Table II and the blood chemistry data in Table III. Liver and kidney function tests were conducted before and after treatment.

Since fatty changes were observed in the livers of the dogs and monkeys of the previous study (2, 17), the lipide fractions in the livers of the dogs were determined one week after the terminal streptomycin injection. The alcohol-ether-soluble fraction of the livers of the two dosed dogs constituted 20% and 27.5% of the dry weight of the liver. The untreated dogs had 21.6% and 23.0% lipide in their livers. Further analyses showed that the lipide patterns were not affected. The results, expressed as per cent of the total lipide of the liver, appear in Table V.

In general the foregoing data indicate that the dogs which received streptomycin for 20 days were practically unaffected by the treatment. The only changes which might be considered significant were (1) a decreased excretion of indican, possibly due to an effect of streptomycin

<sup>3</sup> We wish to thank Drs. F. J. Wolf and R. G. Denkwalter for these preparations.

TABLE II  
*Urinary Analyses<sup>1</sup>*

Dogs 769 and 829 received 100 mg. streptomycin/kg./day subcutaneously for 20 days

Dog No.	Urine volume, <sup>2</sup> ml				Specific gravity <sup>2</sup>				pH <sup>2</sup>			
	Cont.	7 days	14 days	21 days	Cont.	7 days	14 days	21 days	Cont.	7 days	14 days	21 days
769	147	129	153	151	1.043	1.048	1.043	1.049	6.7	6.1	6.2	6.2
767	197	213	180	88	1.047	1.046	1.053	1.060	6.9	6.4	6.9	6.6
829	158	104	169	139	1.053	1.053	1.038	1.044	6.7	6.1	6.3	6.1
772	198	183	158	183	1.044	1.037	1.039	1.044	7.1	6.5	6.8	6.5
	Protein, <sup>2</sup> g./l.				Reducing substances <sup>3</sup> as mg. glucose				Creatine, <sup>4</sup> mg.			
	Cont.	7 days	14 days	21 days	Cont.	7 days	14 days	21 days	Cont.	7 days	14 days	21 days
769	0.69	0.93	1.08	0.58	685	875	790	1030	89	190	210	225
767	0.55	0.50	0.41	0.31	605	660	570	725	25	205	165	180
829	0.63	0.34	0.41	0.38	535	880	855	1410	57	215	185	140
772	0.49	0.50	0.42	0.36	585	580	560	480	30	85	190	65
	Total N, <sup>2</sup> g.				Urea N, <sup>2</sup> g.				Ammonia N, <sup>1</sup> mg.			
	Cont.	7 days	14 days	21 days	Cont.	7 days	14 days	21 days	Cont.	7 days	14 days	21 days
769	6.47	7.82	7.02	7.57	4.75	6.35	5.00	6.07	185	210	215	175
767	4.45	5.75	5.01	6.85	3.17	4.58	3.81	4.87	210	195	175	180
829	4.78	7.32	6.20	7.65	3.68	6.00	4.95	6.23	155	130	110	105
772	5.82	6.93	7.18	5.32	4.68	5.33	5.33	4.31	165	330	—	205

<sup>1</sup> The benzidine test for blood remained negative throughout. Other qualitative changes were a decreased excretion of indican and a less intense color development in Pettenkofer's test for bile salts in the urines of the dosed dogs. No consistent change was detected in bile pigment. Dog No. 769 gave a more intense coloration in the test for urobilinogen during the experimental period.

<sup>2</sup> Averages of 4-7 overnight samples per week.

<sup>3</sup> Determinations made once weekly and calculated in mg. or g./300 mg. creatinine.

excreted in the bile (2), an increase in urinary reducing substances, due to the reducing properties of the streptomycin excreted in the urine, and (3) approximately 20% more fat in the liver of one dog than in that of the control dog.

In a subsequent experiment, to be reported in detail elsewhere, two young beagle hounds weighing 5.6 and 5.2 kg., respectively, were injected subcutaneously for two weeks with 300,000 units/kg./day of a preparation of streptomycin calcium chloride complex<sup>3</sup> (90% pure). No biochemical abnormalities were detected in these dogs, but the food intake was decreased.

## DISCUSSION

The histamine-like factor (perhaps histamine itself) present in various lots of impure streptomycin is at least partially responsible for certain unfavorable reactions found in man and in animals. Histamine-like effects were so prevalent with early lots of streptomycin that the Food and Drug Administration has prescribed that each lot must pass a blood pressure test in cats before it may be released for clinical use.

TABLE III  
*Blood Chemistry<sup>1</sup>*

Dog No	Control	1 week	2 weeks	3 weeks	Control	1 week	2 weeks	3 weeks
Serum protein, g.-%					Total plasma cholesterol, mg.-%			
769	6.3	6.5	6.1	5.9	133	121	125	93
767	6.5	7.6	7.3	6.8	123	123	180	121
829	5.5	5.7	6.2	5.8	113	93	95	82
772	5.8	5.5	5.4	5.8	130	118	112	107
Albumin-globulin ratio					Free plasma cholesterol, mg.-%			
769	1.26	0.92	1.12	1.2	21	26	22	19
767	0.48	0.38	0.52	0.56	30	25	30	33
829	1.45	1.17	1.22	1.0	17	17	18	19
772	1.23	1.28	1.65	1.6	25	14	25	20
Plasma fibrinogen, mg.-%					Cholesterol ester—total cholesterol ratio			
769	260	360	310	280	0.85	0.78	0.82	0.80
767	190	230	180	230	0.75	0.80	0.83	0.69
829	210	330	250	230	0.85	0.82	0.81	0.74
772	360	290	270	290	0.81	0.88	0.78	0.79
Blood urea N, mg.-%					Total blood lipides, per cent			
769	8.8	9.1	15.1	10.5	1.07	1.12	1.22	1.26
767	7.0	8.4	10.2	10.2	1.12	1.25	1.29	1.28
829	7.0	13.7	12.3	13.0	1.08	1.11	1.16	1.18
772	13.3	13.7	13.0	11.9	1.15	1.19	1.16	1.27

<sup>1</sup> 769 and 829 injected, 767 and 772 controls.

TABLE III—*Continued*

Dog No.	Control	1 week	2 weeks	3 weeks	Control	1 week	2 weeks	3 weeks
Blood NPN, mg.-%					Serum phosphatase (units)			
769	27	41	37	34	0.97	0.25	0.75	0.60
767	30	34	31	31	0.70	0.55	0.85	0.45
829	31	37	36	37	0.80	0.45	0.55	0.25
772	35	35	35	37	0.77	0.25	0.70	0.50
Plasma creatinine, mg.-%					Serum inorganic phosphorus, mg.-%			
769	0.85	0.76	0.88	0.88	2.93	3.0	2.9	2.8
767	0.85	0.76	0.82	0.93	2.20	2.15	2.2	2.35
829	0.83	0.70	0.75	0.88	2.10	2.35	2.15	2.8
772	0.85	0.70	0.82	0.88	1.75	1.95	1.7	2.3
Plasma creatine, mg.-%					Serum chloride, meq./l.			
769	0.87	0.88	1.27	0.87	108	106	104	104
767	0.60	0.69	0.56	0.31	108	107	105	105
829	0.83	1.13	0.81	1.12	103	106	109	102
772	0.81	0.70	0.73	0.35	109	115	109	111
					Blood sugar, mg.-%			
					78	100	90	85
					90	100	90	96
					74	90	90	71
					79	100	90	86

Improved manufacturing procedures have largely eliminated this factor at the present time.

The presence of another impurity (or impurities) was revealed by the intravenous toxicity test in mice (2). This test has also been required of all clinical streptomycin. Again, improved chemical procedures have resulted in products of lower toxicity in mice.

Another effect of streptomycin is a neurotoxic effect, which is observed in both man (18) and animals (2, 17). Recent investigations by Molitor and Kuna (19) indicate a possible correlation between the acute neurotoxic effect in rabbits and the acute intravenous toxicity

TABLE IV  
*Liver and Kidney Function Tests<sup>1</sup>*

Diuresis—concentration test

Dog No.	Water dose		Overnight urine				2 hr. pre-test urine			
	Control	Test	Control		Test		Control		Test	
			cc.	sp.gr.	cc.	sp.gr.	cc.	sp.gr.	cc.	sp.gr.
769	315	295	440	1.013	270	1.027	160	1.006	215	1.003
767	375	360	640	1.022	315	1.036	335	1.004	260	1.003
829	350	305	330	1.026	185	1.048	310	1.009	265	1.007
772	360	365	315	1.015	305	1.034	300	1.008	300	1.002

0-4 hr. urine				4-8 hr. urine				8-24 hr. urine			
Control		Test		Control		Test		Control		Test	
cc.	sp.gr.	cc.	sp.gr.	cc.	sp.gr.	cc.	sp.gr.	cc.	sp.gr.	cc.	sp.gr.
380	1.002	370	1.005	60	1.016	37	1.017	67	1.030	80	1.025
380	1.003	405	1.003	35	1.028	20	1.030	55	1.048	45	1.041
305	1.002	310	1.003	27	1.030	31	1.022	35	1.042	70	1.030
375	1.003	445	1.003	38	1.021	78	1.016	62	1.038	145	1.020

Urea clearance and phenolsulfonphthalein excretion

Dog No.	Blood urea N, mg.-%		Mg. urea N in 0-4 hr. urine		Cc. blood cleared of urea per minute		Per cent PSP. in 0-4 hr. urine <sup>2</sup>	
	Control	Test	Control	Test	Control	Test	Control	Test
769	6.3	5.2	330	352	22	29	80	62
767	8.1	5.6	480	640	25	48	75	60
829	6.7	5.5	466	310	29	24	71	57
772	10.2	6.9	519	490	21	29	70	60

Bromsulfalein retention<sup>3</sup>

Dog No.	Per cent retained after 5 min.		Per cent retained after 15 min.	
	Control	Test	Control	Test
769	20	5	0	0
767	5	20	0	0
829	20	10	0	0
772	10	10	0	0

<sup>1</sup> No. 769 and No. 829 injected, No. 767 and No. 772 controls.

<sup>2</sup> No PSP. detected in 5-8 hr. urines.

<sup>3</sup> The thymol turbidity test, carried out weekly, also showed no change with all tests less than 0.5 unit.



TABLE V  
*The Pattern of Liver Lipides in Dogs Injected with  
 Streptomycin<sup>1</sup> for 25 days*

Dog No.	Free cholesterol	Cholesterol esters	Total fatty acids	Phospholipide	Neutral fat
769	2.32	1.68	43.8	32.2	27.8
767	2.23	1.64	41.3	25.7	23.1
829	2.14	1.51	40.0	23.5	23.3
772	2.42	1.29	38.0	24.0	21.7

<sup>1</sup> No. 769 and No. 829 injected, No. 767 and No. 772 controls.

in mice, which suggests a relationship between the factors responsible. Although streptomycin of high potency produces this neurotoxic effect in rabbits, there is little doubt that impurities may in part contribute to both the acute neurotoxicity and the acute intravenous toxicity.

In view of the relative freedom from biochemical changes with more highly purified streptomycin in the dog and the variable effects of impure lots, it seems likely that impurities of different nature contributed to the biochemical effects of streptomycin in the dogs of the previous experiments. In this connection it is of interest that the 4 dogs which received the more highly purified streptomycin failed to show the disturbance in equilibrium observed after injection of low potency material, although the same amount or more of the antibiotic in units/kg. was administered.

In line with the above findings in dogs, as more satisfactory material became available for clinical use, the early indications of renal impairment following streptomycin therapy in man gave way to more favorable reports. In studies with low potency experimental lots of streptomycin conducted with typhoid carriers, Rutstein *et al.* (20) reported evidence of kidney insufficiency after the intramuscular injection of about 3,000,000 units over a 3 day period. More recent lots of streptomycin have been administered in larger amounts and for longer periods of time without serious toxic effects on the kidney. The drug has been given to patients with tuberculosis of the kidney without renal complications (18, 21). Other investigators have likewise reported no renal complications resulting from streptomycin therapy in man (22, 23, 24, 25, 26). The latter two groups of investigators per-

formed tests of liver function in their subjects and found no impairment.

In conducting biochemical studies in animals or men receiving streptomycin, one must remember that streptomycin itself has a reducing effect equivalent to about 30% its weight of glucose. Consequently, a positive test for sugar may result from the streptomycin excreted in the urine. Moreover, when such precipitating agents as phosphotungstic acid (*i.e.*, Tsuchiya's solution) are used in the test for urinary protein, a false positive test may be obtained, particularly when low potency streptomycin has been administered.

#### ACKNOWLEDGMENT

Thanks are due Mr. John Flanagan for technical assistance.

#### CONCLUSIONS

1. When impure streptomycin was administered daily for 5–20 days to dogs and monkeys at levels of 25,000–200,000  $\gamma$  (or units)/kg., kidney and liver complications developed in some of the animals. This was evidenced by bromsulfalein retention and proteinuria. These effects disappeared when treatment was discontinued.

2. In contrast to the above results, when streptomycin in a relatively pure form was injected subcutaneously into dogs for 20 days at a level of 100,000  $\gamma$  (or units) /kg., no significant changes in food intake, body weight, liver lipides, blood and urine chemistry, and liver and kidney function, were observed. No significant biochemical effects were observed in two dogs which were injected subcutaneously daily for two weeks with 300,000 units/kg. of a streptomycin calcium chloride complex which was 90% pure. The only effect found was a decrease in food intake. The evidence suggests that the effects found with early lots of streptomycin can be attributed, at least in part, to impurities.

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# Storage and Distribution of Vitamin A in Rats Fed Certain Isomers of Carotene \*

R. M. Johnson and C. A. Baumann

*From the Department of Biochemistry, College of Agriculture,  
University of Wisconsin, Madison*

Received April 10, 1947

## INTRODUCTION

Most investigators apparently regard the biological activity of the carotenoids as dependent upon their conversion into vitamin A in the animal. Recently, however, With (1) has raised the question whether, in certain species at least, carotenoids might not themselves possess vitamin activity without first being converted into vitamin A. His view is based on calculations which indicate that cryptoxanthine is roughly twice as active as  $\beta$ -carotene in the chick (2), in contrast to the greater activity of the latter pigment in other species (3, 4, 5). In the present study an approach was made to the question of carotenoid activity *per se* by determining the amounts of vitamin A in the tissues of rats fed carotenoids of various degrees of growth-promoting activity. The compounds included all-trans- $\beta$ -carotene, neo- $\beta$ -carotene B, neo- $\beta$ -carotene U, and all-trans- $\alpha$ -carotene.

## MATERIALS AND METHODS

$\beta$ -Carotene and  $\alpha$ -carotene were prepared from a commercial crystalline carotene by adsorption on a mixture of one part of Micon Brand MgO, Lot 2641<sup>1</sup>, with one part of Hyflo Super-Cel.<sup>2</sup> The chromatograms were developed with petroleum ether (Skelly Solv B). Each preparation was rechromatographed until it appeared homogeneous, and the pigments were finally recrystallized from benzene-ethanol. The purity of the compounds was determined in a Beckman spectrophotometer.

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\* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This investigation was aided by grants from the Jonathan Bowman Fund for Cancer Research and from the Wisconsin Alumni Research Foundation.

<sup>1</sup> Magnesium Oxide obtained from Westvaco Chlorine Products Co., Newark, California.

<sup>2</sup> Hyflo Super-Cel obtained from Johns-Manville and Company, Chicago, Illinois.

Neo- $\beta$ -carotene B and neo- $\beta$ -carotene U were prepared from pure  $\beta$ -carotene as follows (6): the carotene was dissolved in Skelly Solv B, iodine was added equivalent to 2% of the weight of the carotene, and the solution was exposed for 60 minutes to a 100-watt incandescent light at approximately 20 cm. The solution was then washed 4 times with small portions of a  $N/10$   $Na_2S_2O_4$  solution followed by 3 washings with distilled water, after which it was dried over anhydrous  $Na_2SO_4$ . The two neo-carotenes were separated from other carotenoids on a mixture of 3 parts of Merck's reagent grade  $Ca(OH)_2$  and one part of Hyflo Super-Cel; the developing solution consisted of 0.5% acetone in Skelly Solv B. This did not, however, result in a separation of isomers occurring above neo- $\beta$ -carotene U on the column. The pigments were recrystallized from benzene-ethanol and their purity determined spectrophotometrically by comparison with values published by Zechmeister and co-workers (6, 7). The various carotenoids were fed in cottonseed (Wesson) oil solution containing the desired daily supplement and 0.5 mg. of  $\alpha$ -tocopherol in 3 drops of the oil as measured from calibrated droppers. Fresh supplements were prepared every 4 days, and stored in the refrigerator.

Weanling Sprague-Dawley rats were placed on a diet consisting of vitamin A-free casein 18, dextrin 65, Wesson oil 5, brewers' yeast 8, and salts (8) 4. Sixteen drops of Drisdol<sup>3</sup> containing 4000 units of vitamin D were added per 10 kg. of diet. The rats were maintained on this diet until they failed to gain weight over a period of 7 days. Such depletion periods ranged from 24–30 days. The depleted rats were divided into groups equivalent in weight and sex and each group was given 10–80  $\gamma$  daily of the various carotenoids (Table I) for a period of 15 days. The animals were killed by decapitation 24 hours after the last supplement had been given. Whole livers and pairs of kidneys were removed, freed of foreign tissue, and placed in approximately 35 ml. of an 8% aqueous KOH solution. The samples were stored in the dark at room temperature for 17–24 hours, at which time the organs were completely digested. They were then extracted with 3 portions of diethyl ether, and the ether extracts pooled and washed 3 times with cold water and once with a saturated NaCl solution. The ether was removed by evaporation under reduced pressure at a temperature of 40–45°C. The flasks were washed down with a few ml. of  $CHCl_3$ , and again evaporated to dryness. The residues were finally taken up in  $CHCl_3$ , and vitamin A and "carotene" determined colorimetrically on suitable aliquots (9, 10). Only negligible amounts of vitamin A were present in lungs, heart, adrenals, and spleen under the conditions of this experiment.

## RESULTS

At the time of depletion, the animals were emaciated and usually showed eye symptoms characteristic of avitaminosis A, and analysis of the livers and kidneys of 8 rats indicated that no vitamin A was present at this time. After administration of the various carotenoids all animals appeared to be normal. While the amounts of the caro-

<sup>3</sup> A crystalline Vitamin D concentrate obtained from Winthrop Chem. Co., Inc., New York, N. Y.

tenoids fed were in excess of those usually considered optimal for growth, there was a tendency toward faster growth on the larger amounts of supplement given (Table I).

TABLE I  
*Conversion of Various Biologically Active Carotenoids  
to Vitamin A in the Rat \**

Supplement	Dose per day	No of rats	Weight gains	Kidney		Liver		Total "Caro- tene"	Total Vit. A
				"Caro- tene"	Vit. A	"Caro- tene"	Vit. A		
All-trans- $\beta$ - carotene	10	4	44	0.92	2.1	3.0	0	3.92	2.1
	20	8	50	1.2	17.5	4.8	13.1	6.0	30.6
	40	8	65	1.1	29.5	5.2	38.8	6.3	68.3
Neo- $\beta$ - carotene B	22.5	4	—	.79	12.8	5.5	2.8	6.1	15.6
	40	8	51	1.1	14.3	5.6	14.6	6.7	28.9
	80	8	67	1.2	20.0	6.0	23.4	7.2	43.4
Neo- $\beta$ - carotene U	40	8	45**	.92	14.0	6.5	5.9	7.4	19.0
	80	8	53	1.1	19.9	6.1	18.8	7.2	38.7
All-trans- $\alpha$ - carotene	13	4	—	1.5	1.94	7.4	0	8.9	1.94
	40	8	52	0.77	8.2	6.4	1.0	7.17	9.2
	80	8	40	0.92	14.1	7.1	16.7	8.32	30.8

\* Figures given represent means of the total amounts in the liver or in the pairs of kidneys. "Carotene" is the total ether-soluble yellow pigment present. Weight gains cover the fifteen day supplementation period.

\*\* Mean of four animals.

There was no evidence of any accumulation of carotenoids in either the liver or the kidneys when the various isomers of carotene were fed. Ether-soluble yellow pigments were present equivalent in intensity to 3.0–7.4  $\gamma$  of carotene per liver and 0.8–1.5  $\gamma$  per pair of kidneys (Table I). However, fractionation of such extracts on MgO indicated that the yellow materials moved much more rapidly with petroleum ether than either  $\alpha$ - or  $\beta$ -carotene. Furthermore, the total amounts of ether-soluble yellow were essentially the same whether a high amount of carotene or none at all was fed. Hence, it was concluded that the yellow materials were not involved in the formation or storage of vitamin A.

For all isomers an increase in the intake of carotene resulted in an increase in vitamin A storage in the body, while the distribution of the vitamin between tissues varied with the amounts of carotenoid fed. At very low levels of intake the kidney frequently stored vitamin A, although none could be demonstrated in the liver. This occurred, *e.g.*, when either 10  $\gamma$  of  $\beta$ -carotene or 40  $\gamma$  of  $\alpha$ -carotene were fed daily for 15 days; on the latter level, kidney vitamin A values in four animals ranged from 7.9 to 18.7  $\gamma$  (Table I). The amounts of vitamin A stored in the kidney equalled or exceeded those in the liver over fairly wide ranges of carotene intake, *viz.*, daily intakes up to 20  $\gamma$  of all-trans- $\beta$ -carotene, to 40  $\gamma$  of neo- $\beta$ -carotene B, or to 80  $\gamma$  of neo- $\beta$ -carotene U or of all-trans- $\alpha$ -carotene (Table I). At higher levels of administration, however, liver storage exceeded kidney storage.

The distribution of vitamin A between tissues appeared to depend upon the total amount in the body rather than upon the compound in the diet from which it was derived. This was shown in another experiment in which small amounts of halibut oil were diluted with corn oil and fed to depleted rats for 15 days. As when carotenoids were fed, kidney storage equalled or exceeded liver storage at low levels of administration, *e.g.*, 10 or 20 I.U. daily, but when 30 or 40 I.U. were fed daily, the amounts in the liver exceeded those in the kidney.

The total amounts of vitamin A stored in the liver and kidneys as a result of feeding the various isomers of carotene indicated that the order of decreasing activity for effecting storage is all-trans- $\beta$ -carotene > neo- $\beta$ -carotene B > neo- $\beta$ -carotene U > all-trans- $\alpha$ -carotene. When 20  $\gamma$  of all-trans- $\beta$ -carotene were fed daily, the total amount of vitamin A in the liver and kidneys average 30.6  $\gamma$ . When neo- $\beta$ -carotene B was fed, however, 40  $\gamma$  per day were required to produce average stores of 28.9  $\gamma$ , while the daily dose of neo- $\beta$ -carotene U necessary to produce a comparable storage lay between 40 and 80  $\gamma$ .  $\alpha$ -Carotene was the least active compound in producing vitamin A stores: a dosage of 80  $\gamma$  per day was required before the stores of vitamin A reached 30.8  $\gamma$  (Table I). Incidentally, the preparation of  $\alpha$ -carotene fed had a specific extinction coefficient of 266 at 4465 Å in Skelly Solv B. Zscheile *et al.* report values of 272–273 in hexane (11). The growth-promoting power of this preparation of  $\alpha$ -carotene, in agreement with results of Deuel and associates (12), proved to be at least half that of  $\beta$ -carotene when fed to rats at levels of 1.8 and 3.0  $\gamma$  daily.

## DISCUSSION

The amounts of vitamin A stored in the body represent an excess above those used to meet the daily needs of the organism, and it is generally agreed that the border dose for storage exceeds that needed to prevent symptoms of avitaminosis by a factor of about 10 (13, 14, 15). However, if the carotenoid is devoid of activity *per se* and functions only as a source of vitamin A, the same amount of vitamin would be used up daily regardless of which precursor was fed, and comparative biological activities of the various precursors could be calculated from the dosages needed to produce stores of comparable magnitude. The following items from Table I then would become pertinent:

20 $\gamma$ all-trans- $\beta$ -carotene daily	30.6 $\gamma$ of stored vitamin A
40 $\gamma$ neo- $\beta$ -carotene B	28.9 $\gamma$ of stored vitamin A
60 $\gamma$ neo- $\beta$ -carotene U	30.05 $\gamma$ of stored vitamin A (av.)
80 $\gamma$ $\alpha$ -carotene	30.8 $\gamma$ of stored vitamin A

If the biological activity of all-trans- $\beta$ -carotene is set at 100, the activity of any isomer will be

$$\frac{\text{dose of all-trans-}\beta\text{-carotene} \times \text{storage due to isomer} \times 100}{\text{dose of isomer} \times \text{storage due to } \beta\text{-carotene}}$$

For neo- $\beta$ -carotene B the activity will be  $\frac{20 \times 28.9 \times 100}{40 \times 30.6} = 48$ ; for neo- $\beta$ -carotene U it will be 33 and for  $\alpha$ -carotene, 25. Considering the assumptions made, and the possibility of unequal losses of the different carotenoids due to incomplete intestinal absorption of the relatively high dosages needed for storage as compared to the low dosages used in the growth experiments, the results agree satisfactorily with the growth-promoting activities of 100:53:38 reported for all-trans- $\beta$ -carotene, neo- $\beta$ -carotene B, and neo- $\beta$ -carotene U respectively (16). In other words, the growth-promoting powers of the stereoisomers parallel their abilities to produce stores of vitamin A in the liver and kidney of the rat.

$\alpha$ -Carotene, however, appears to be less efficient in producing stores of vitamin A in the body than in promoting growth in rats. Present storage experiments indicate  $\alpha$ -carotene to be only  $\frac{1}{4}$  as effective as  $\beta$ -carotene, a conclusion that can also be drawn from the data of Brockmann and Tecklenburg (17), in contrast to an activity of 53%



relative to  $\beta$ -carotene (12) when growth is the basis of comparison. The cause of the discrepancy, however, is not clear from the present results. One possibility is that certain carotenoids might possess biological properties *per se*, as suggested by With (1), and that there is no universal parallelism between the abilities of the various pigments to promote growth and to produce stores of vitamin A. In the present experiments the parallelism between growth and storage due to the stereoisomers of  $\beta$ -carotene might suggest that their activities depend upon the degrees to which the various isomers can be converted to all-trans- $\beta$ -carotene in the body (18)

The preferential accumulation of small amounts of vitamin A in the kidney rather than in the liver requires some comment. Apparently most workers who studied the distribution of vitamin A in various organs used animals that had been fed sufficiently large amounts of carotenoids (19) or vitamin A (13, 20) so that liver storage exceeded kidney storage. If lesser amounts of carotenoid had been fed, *e.g.*, in the original experiments of Moore (19), the suggestion may well have emerged that carotene was converted to vitamin A in the kidney! A preferential accumulation of vitamin A in kidney has also been observed in rats fed crude carotenoid mixtures containing chlorophyll (21)

#### SUMMARY

1.  $\alpha$ -Carotene,  $\beta$ -carotene, and two of its stereoisomers, neo- $\beta$ -carotene B, and neo- $\beta$ -carotene U, were fed to depleted rats for 15 days in amounts of 10–80  $\gamma$  per day, and the stores of vitamin A produced in the liver and kidneys were determined colorimetrically.

2. At low levels of carotene intake, 35 I.U. daily, more vitamin A appeared in the kidney than in the liver; at higher levels of intake more vitamin appeared in the liver than in the kidney.

3. The relative abilities of the isomers to promote vitamin storage were as follows: all-trans- $\beta$ -carotene 100, neo- $\beta$ -carotene B 48, neo- $\beta$ -carotene U 33, all-trans- $\alpha$ -carotene 25. Thus the amounts of vitamin A stored varied with the known capacities of the stereoisomers of  $\beta$ -carotene to promote growth in depleted animals.  $\alpha$ -Carotene, however, yielded lower stores of vitamin A than its growth-promoting power would lead one to expect.

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# The Utility of Bios Response in Yeast Classification and Nomenclature

Alfred S. Schultz and Lawrence Atkin\*

*From the Fleischmann Laboratories, Standard Brands, Inc., New York, N. Y.*

Received April 24, 1947

## INTRODUCTION

It has become increasingly apparent that characterizing yeasts according to their bios requirements would prove valuable as a supplement to present morphological and physiological methods of classification. The classical methods of the taxonomist are inadequate for the differentiation of yeasts within a species. Variably named strains of yeast of the same species frequently are similar morphologically, and in their abilities to ferment sugars. Differences in bios requirements provide a firm basis for subclassification or integration of such strains.

Schultz, Atkin, and Frey (1) originally published a biochemical method for subclassifying strains, varieties and races of *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis* yeasts into 3 types, designated A, B, and C. The classification was based upon crop values of yeasts in a basal medium which included inositol,  $\beta$ -alanine and bios II-B, and in the same medium after respective additions of thiamine, and of thiamine plus pyridoxine. A later paper by the same authors (2) described a rapid method for measuring yeast growth by light absorption measurements. This method simplified the procedure of biochemically classifying yeasts.

In general, 6 well-defined bios factors have been established. These are inositol, pantothenic acid, biotin, thiamine, pyridoxine, and nicotinic acid. Not all of these factors were available in purified forms when our earlier studies on the biochemical classification of yeasts were published (1 and 2). After crystalline calcium pantothenate and biotin became available, yeast growth factor studies have been reported by

\* Present address: Wallerstein Laboratories, 180 Madison Avenue, New York 16, N.Y.

Burkholder (3), Burkholder and Moyer (4), Burkholder, McVeigh and Moyer (5), Leonian and Lilly (6), Lochhead and Landarkin (7), Rogosa (8), and Emory, McLeod and Robinson (9).

It has thus become more and more evident that by establishing the growth factor requirements of yeasts we provide an identifying characteristic which can be a valuable supplement in any system of classifying these microorganisms. Furthermore, if growth factor requirement data are to be broadly and most advantageously used in differentiating yeast strains, a universally acceptable system of recording these data should be adopted. The purpose of this article is twofold: first, to present one procedure which we have used to determine the growth factor response of yeasts, together with data obtained by applying this procedure to a number of strains of *Saccharomyces* and other genera; and secondly, to propose a simple system of recording this response in the form of a "bios number" which is a characteristic index of strain response. The bios number is simply an index which defines the growth factor requirement of definite yeast strains as herein determined.

### EXPERIMENTAL

Nine tests are required to determine growth factor responses of a yeast by the following procedure. The yeast is grown in a synthetic medium (basal medium) containing ammonium sulfate as the only source of nitrogen, and the 6 growth-promoting agents (inositol, calcium pantothenate, biotin, thiamine, pyridoxine, and nicotinic acid); in the basal medium containing casein hydrolyzate and the 6 growth-promoting agents; and in the basal medium containing casein hydrolyzate and all but 1 of the 6 growth factors, except in one instance where both  $B_1$  and  $B_6$  are omitted. This yeast culture procedure is detailed below.

#### *Preparation of Solutions*

Make up the following solutions in sufficient amounts for the number of yeast cultures to be typed. In each case, distilled water is used as the solvent.

Solution	Concentration	Sterilization
Ammonium sulfate	75 g. $(\text{NH}_4)_2\text{SO}_4$ /1000 ml.	Flowing steam 30 minutes on 3 successive days.

Solution	Concentration	Sterilization
Inositol	0.5 g./1000 ml.	Flowing steam 30 minutes on 3 successive days.
Calcium pantothenate	0.2 g./1000 ml.	Do not sterilize. Store in refrigerator.
Biotin	100 mγ/ml.	Flowing steam 30 minutes on 3 successive days.
Thiamine hydrochloride (B <sub>1</sub> )	10 γ/ml.	Flowing steam 15 minutes.
Pyridoxine hydrochloride (B <sub>6</sub> )	10 γ/ml.	Flowing steam 30 minutes on 3 successive days. Store in dark.
Nicotinic acid	10 γ/ml.	Flowing steam 30 minutes on 3 successive days.
Sugar and salts (SAS) solution	KH <sub>2</sub> PO <sub>4</sub> 17.6 g. KCl 13.6 g. CaCl <sub>2</sub> ·2H <sub>2</sub> O 4.0 g. MgSO <sub>4</sub> ·7H <sub>2</sub> O 4.0 g. FeCl <sub>3</sub> 0.08 g. MnSO <sub>4</sub> 0.08 g. Dextrose 1600.0 g. Dissolve salts separately and add together in the above order. Dextrose is added as is, with constant shaking. Make total volume to 8 liters. Store in five 2-liter Erlenmeyer flasks.	Flowing steam 30 minutes on 3 successive days.
Potassium citrate buffer	Potassium citrate K <sub>3</sub> C <sub>6</sub> H <sub>5</sub> O <sub>7</sub> ·H <sub>2</sub> O, 100 g. Citric acid H <sub>3</sub> C <sub>6</sub> H <sub>5</sub> O <sub>7</sub> ·H <sub>2</sub> O, 20 g. Dissolve and adjust pH to 5.2. Make volume to 1000 ml.	Flowing steam 30 minutes on 3 successive days.
Saline	NaCl, 18 g./2000 ml. (0.9%). Store in Erlenmeyer flasks and in 10 ml. amounts in test tubes plugged with cotton.	Steam autoclave at 15 pounds pressure for 15 minutes.
Agar slants	Bacto agar 20-25 g./1000 ml. Blackstrap molasses 20 g./1000 ml. Difco malt extract 45 g./1000 ml.	Steam autoclave at 15 pounds pressure for 15 minutes.

*Acid-Hydrolyzed Casein Solution*

Mix 100 g. of vitamin-free casein with 500 ml. of constant boiling HCl (approximately 20% HCl—250 ml. of 38% HCl and 250 ml. H<sub>2</sub>O) and reflux the mixture for 8 hours. Remove the HCl from the refluxed mixture by distillation under reduced pressure until a thick syrup remains. A NaOH trap may be used to collect the acid. Dissolve the syrup in 200 ml. of distilled water and concentrate the mixture again in the same manner. Redissolve the resulting mixture in distilled water. Adjust the pH to 3.0 with NaOH and add sufficient H<sub>2</sub>O to make no more than 950 ml. Add to the solution 20 g. of activated charcoal (Darco G-60) and stir for 1 hour. Filter. Repeat the treatment with activated charcoal if the filtrate does not appear straw-colored to colorless. Adjust the pH of the filtrate to 6.8 and add sufficient distilled H<sub>2</sub>O to bring the volume to 1000 ml. Store this solution under toluene in the refrigerator.

*Basal Medium*

Make up the basal medium with the following proportions:

SAS	160 ml.
Citrate buffer	32 ml.
Distilled water	128 ml.

Arrange in racks, numbered test tubes (18 × 150 mm.) calibrated for absorption (2). Nine test tubes are needed for each yeast culture. Add 5 ml. of the basal medium to each tube. To the No. 1 tube in each series add 0.5 ml. of ammonium sulfate solution, and to tubes No. 2-9 add 0.5 ml. of casein hydrolyzate. Add 0.5 ml. of each growth factor solution (inositol, calcium pantothenate, biotin, B<sub>1</sub>, B<sub>6</sub>, niacin) to tubes No. 1 and 2. Tubes No. 3-9 should receive 0.5 ml. additions of each of these growth factor solutions except for the growth factor or factors indicated for specific omission from individual tubes under the section entitled "Determination of Bios Numbers." Bring the final total volume of each tube up to 9.5 ml. with distilled H<sub>2</sub>O. Plug the tubes with cotton, sterilize by heating in flowing steam for 15 minutes and cool rapidly in water bath.

*Yeast Inoculum*

About 1 hour before the end of the working day prepare the yeast inoculum using a fresh 24-hour growth at 30°C. on malt extract-molasses agar. Transfer by needle to a tube containing 10 ml. of sterile saline, sufficient yeast to give a Lumetron reading of 20% absorption of light. This should amount to approximately 1 mg. of moist yeast/ml. If a slight adjustment is necessary to obtain this concentration, it may easily be made at this time. Then, add the contents of the tube to a 125 ml. Erlenmeyer flask containing 40 ml. of sterile saline, giving a total volume of 50 ml.

Using a sterile 5 ml. graduated pipet for each yeast, transfer 0.5 ml. of the suspension to each of the 9 test tubes containing the media previously prepared. This amount of inoculum is equivalent to about 0.1 mg. of moist yeast per tube.

*Yeast Growth*

In each inoculated test tube, turn under and push down the cotton plugs so they are tight and there are no loose cotton ends. Put the tubes on shaker blocks and shake in an incubator maintained at  $30^{\circ} \pm 0.5^{\circ}\text{C}$ . A Fisher-Kahn shaking apparatus or its equivalent may be used, provided shaking is sufficient to prevent the yeast from settling. After an incubation period of 24 hours, measure yeast growth in each tube by reading the percentage absorption of light on a Lumetron 400 colorimeter. If the tube containing all bios factors and casein has not reached an absorption value of 80% in 24 hours continue incubation of the 9 tubes in that series up to 40 hours. The 80% absorption value corresponds to 60 mg. of moist yeast per tube. Yeasts which do not attain an 80% value in 40 hours have not been classified, thus far. If growth in a given medium deficient in one of the bios factors does not reach 50% absorption (30 mg. of yeast per tube), it is taken as evidence that the yeast under test requires that factor which is not present.

In the course of this work and during previous investigations (2) it was determined that yeasts generally grow at a faster rate with casein hydrolyzate than with ammonium sulfate as the nitrogen source. It is for this reason that casein hydrolyzate was chosen for use in the studies herein described. The ammonium sulfate medium was included in order to determine whether any of the yeasts studied were incapable of utilizing ammonium nitrogen under conditions of adequate bios nutrition.

## DETERMINATION OF BIOS NUMBERS

After a yeast has been grown in the 9 test solutions it is given a bios number. The bios number (B.N.) of a culture identifies the factor or factors which do not permit full growth of a culture when individually omitted from a medium otherwise containing the full complement of growth factors. The growth factors used for characterizing yeasts are numbered from 1 to 7, as follows:

1. Inositol
2. Calcium pantothenate
3. Biotin
4. Thiamine ( $B_1$ )
5. Pyridoxine ( $B_6$ )
6. Thiamine and pyridoxine ( $B_1$  and  $B_6$ )
7. Nicotinic acid

Thus, if a yeast has a B.N. of 23, it means that full growth of the culture will not be obtained when a single omission from the medium is made of factor 2 (calcium pantothenate), or of factor 3 (biotin). In numbering the factors, it was found necessary to consider  $B_1$  and  $B_6$  both individually, and in combination (note factors 4, 5, and 6). Whenever 4 or 5 is



used in a B.N. it should be considered exactly as described in the illustration above. B.N. 6 is never used in such instances, but is reserved for situations where full growth of the culture is obtained with single omissions of either B<sub>1</sub> or B<sub>6</sub>, but is not obtained when both are omitted. Cultures which require from 24 to 40 hours for attainment of an 80% absorption value on the complete medium containing all bios factors and casein are given bios numbers identified by *italics*. Thus, B.N. 2 is differentiated from B.N. 2.

### RESULTS

Table I gives the results of classifying 13 *Saccharomyces cerevisiae* Hansen cultures. Twelve definite types of this species were identified using the proposed method of classification based upon growth factor requirements. One strain, *Saaz Henneberg*, grew so poorly on the basal medium containing casein hydrolyzate and the 6 growth-promoting factors that it could not be classified. Evidently, it is an extremely slow

TABLE I  
*Saccharomyces cerevisiae* Hansen

Culture	Source	Bios Number
Strain <i>Sake</i>	C.B.S.*	2
English brewers' yeast	N.C.T.C. No. 6479	?
Strain <i>anamensis</i>	C.B.S.	23
(Wijk)	C.B.S.	23
Strain <i>alpinus</i>	C.B.S.	23 (B <sub>1</sub> inhibits growth)
Strain <i>bataviae</i>	C.B.S.	123
Variety <i>festinans</i>	C.B.S.	123
Bakers' yeast N.C.T.C. 815	A.T.C.C. No. 2335	234
Var. <i>ellipsoideus</i> , str. Delft II	C.B.S.	236
(O.P. (old process bakers' yeast))	F.L.C.	1234
French wine yeast	A.T.C.C. No. 4116	1235
Race XII	I.f.G.	1236
Strain <i>Saaz Henneberg</i>	C.B.S.	Poor growth

\* The following abbreviations are used in this and subsequent tables:

A.T.C.C. —American Type Culture Collection

C.B.S. —Centraalbureau voor Schimmelcultures (Delft, Holland)

I.f.G. —Institut für Gärungsgewerbe (Berlin, Germany)

• N.C.T.C.—National Collection of Type Cultures (London, England)

N.R.R.L.—Northern Regional Research Laboratory

F.L.C. —The Fleischmann Laboratories Collection

growing yeast, or else additional bios factors exist which are essential to its growth, but which were not contained in this medium. In this series of *S. cerevisiae* Hansen yeasts, a B.N. 23 was further designated as a yeast whose growth is inhibited by B<sub>1</sub>. Such a yeast grows well when both B<sub>1</sub> and B<sub>6</sub> are absent or when both are present, but its growth is inhibited when B<sub>1</sub> is present in the absence of B<sub>6</sub>. It therefore cannot be strictly labeled as a B<sub>6</sub>-requiring yeast and is differentiated from such a yeast by being marked "B<sub>1</sub> inhibits growth."

TABLE II  
*Saccharomyces carlsbergensis*

Culture	Source	Bios Number
Strain <i>Frohberg</i>	C.B.S.	3
Chubut	I.f.G.	23
Var. <i>mandshuricus</i> 1	C.B.S.	235
Var. <i>polymorphus</i>	C.B.S.	1235
A.T.C.C. No. 2345	A.T.C.C.	12367
Var. <i>valdensis</i>	C.B.S.	Poor growth

Table II shows 6 cultures of the species *Saccharomyces carlsbergensis* subdivided according to bios needs. Five different types were distinguished. Variety *valdensis* grew too poorly in the basal medium to permit assignment of a definite bios number.

The classification of 14 other species of yeasts of the genus *Saccharomyces* is given in Table III. Among the new bios numbers in this group

TABLE III  
*Saccharomyces*

Culture	Source	Bios Number
<i>Bailii</i>	Germany	0
<i>Pastorianus</i>	A.T.C.C. No. 2339	3
<i>Chevalieri</i>	N.R.R.I. No. 1346	12
Tokyo (Nakazawa)	C.B.S.	23
<i>Behrensianus</i>	C.B.S.	23
<i>Chodati</i>	Yale University <sup>4</sup>	23 (B <sub>1</sub> inhibits growth)
<i>Globosus</i>	Yale University	34
<i>Logos</i>	Yale University	123
<i>Anomalus Belgicus</i>	Germany	234
<i>Bacillaris</i>	C.B.S.	235
<i>Fragilis</i>	Yale University	237
<i>Exiguus</i>	Germany	2347
<i>Ludwigii</i>	Germany	2357
<i>Uvarum</i>	C.B.S.	Poor growth

<sup>4</sup> Yale University cultures received through the kindness of Dr. P. R. Burkholder.

not found among the *cerevisiae* or *carlsbergensis* species are B.N. 0, 12, 34, 237, 2347, and 2357. The 3 latter yeasts are distinguished by the additional requirement of nicotinic acid.

In the genus *Torula* 8 distinct types were differentiated (Table IV).

TABLE IV  
*Torula*

Culture	Source	Bios Number
<i>Utilis</i>	N.R.R.L.	0
<i>Futcheri</i>	Germany	3
<i>Torulopsis dattila</i>	Yale University	4
<i>Thermantitoeum</i>	C.B.S.	23
<i>Torulaspora fermentati</i>	Saito	37
<i>Colliculosa</i>	C.B.S.	123
<i>Torulopsis stellata</i>	U. of California <sup>+</sup>	134
<i>Cremoris</i>	Iowa State	237

<sup>+</sup> H. J. Phaff, College of Agriculture, University of California.

In Table V, a series of miscellaneous yeasts were found to represent many types. The various species represented may be easily differentiated by their distinctive bios numbers.

TABLE V  
*Yeasts (Miscellaneous)*

Culture	Source	Bios Number
<i>Brettanomyces bruxellensis</i>	Yale University	5
<i>Candida guilliermondii</i>	A.T.C.C. No. 9058	3
<i>Candida pseudotropicalis</i>	Yale University	7
<i>Hansenula lambica</i>	Yale University	0
<i>Hansenula anomala</i> var. <i>robusta</i>	C.B.S.	0
<i>Hanseniaspora valbyensis</i>	C.B.S.	Poor growth
<i>Klockera brevis</i>	Yale University	123457
<i>Mycoderma tannica</i> Asai	C.B.S.	0
<i>Mycoderma lipolytica</i>	Yale University	4
<i>Pichia belgica</i>	Yale University	0
<i>Pichia manchurica</i>	C.B.S.	3
<i>Pichia Kluyveri</i>	Yale University	35
<i>Schizosaccharomyces versatilis</i>	N.R.R.L.	127
<i>Schizosaccharomyces mellacei</i>	Germany	236
<i>Schizosaccharomyces pombe</i>	Yale University	1237
<i>Willia anomala</i>	C.B.S.	0
<i>Willia belgica</i>	University of Lyons <sup>+</sup>	4
<i>Zygosaccharomyces marxianus</i>	C.B.S.	7
<i>Zygosaccharomyces priorianus</i>	Yale University	23
<i>Zygosaccharomyces lactis</i>	Yale University	37

<sup>+</sup> Prof. A. Guilliermond.

A special compilation of nicotinic acid-requiring yeasts of a number of genera is represented in Table VI. They are grouped into lactose- and nonlactose-fermenting yeasts.

TABLE VI  
*Nicotinic Acid-Requiring Yeasts*

Culture	Source	Lactose Fermentation	Bios Number
<i>Zygosaccharomyces lactis</i>	Yale University	+	37
<i>Torula cremoris</i>	Iowa State	+	237
<i>Zygosaccharomyces marxianus</i>	C.B.S.	—	7
<i>Schizosaccharomyces versatilis</i>	N.R.R.L.	—	127
<i>Schizosaccharomyces pombe</i>	Yale University	—	1237
<i>Pombe</i> sp 105	Germany	—	2347
<i>Saccharomyces Ludwigii</i>	Germany	—	2357
<i>S. carlsbergensis</i>	A.T.C.C.	—	12367
A.T.C.C. No. 2345			
<i>Kloekera brevis</i>	Yale University	-	123457

## DISCUSSION

The distinct bios requirements of different yeast strains may be used as a valuable complement to the present yeast classification methods, which are mainly based upon morphology, spore formation, and fermentation of specific sugars. In this paper, 13 distinct types of *Saccharomyces cerevisiae* Hansen, and 6 distinct types of *Saccharomyces carlsbergensis* are identified by bios numbers which designate specific growth factor requirements. For example, a yeast previously referred to as *Saccharomyces cerevisiae* Hansen, variety *ellipsoideus*, strain Delft II, can now be labeled as *Saccharomyces cerevisiae* Hansen B.N. 236.

The proposed method for the subclassification of strains as applied to *cerevisiae* and *carlsbergensis* yeasts by Schultz *et al.* (1) has been greatly extended to include all of the known growth factors in pure forms. Thus, many more types of yeast have been recorded than the previous A, B, C types. It is interesting to note that formerly classified type A yeasts were subdivided into types represented by either B.N. 234 or 236 under this new system. If they required inositol they then were designated as 1234 or 1236. Type B yeasts were subdivided into classes represented by B.N. 23, 23, 123 or 123,, while type C were subdivided into classes represented by B.N. 23 (B<sub>1</sub> inhibits growth), 235,

or 1235. A few exceptions to this correlation between previous and present methods of classifying according to bios requirements are due to the impure forms of the growth factors previously used and to low crop values which were often obtained in the former study.

In 1944, Thorne (10) stated, "There is not yet any recognized subdivision of the species *S. cerevisiae* Hansen, so for the time being yeast 6479 must be left without any further label." It is true that at the time, no generally accepted method for differentiating within a species was existent. Table I shows that now subdivision of species has become possible. Furthermore, the particular English top-fermentation brewery yeast referred to by Thorne was obtained from the National Collection of Type Cultures as No. 6479. In a 40-hour test, this yeast was found to require calcium pantothenate as a growth factor, and was consequently designated as B.N. 2. The strain *Sake* (Table I) is also a pantothenate-requiring yeast, but it grew fast enough to type in 24 hours and is designated as B.N. 2.

Although classifying yeasts over a period of approximately 10 years has indicated the permanency of their bios numbers, alterations in growth rate can occur. The condition of the yeast inoculum, variations in the amount of inoculum, and degeneration caused by aging all combine in this effect.

Among the most practical and most frequently occurring strains of *S. cerevisiae* Hansen yeasts are those having B.N. 23, 234, or 236. Strains of these 3 classes have been used over a period of years as bakers' or distillers' yeasts. The identification of such yeasts by classical means was difficult but they may now be distinguished by their bios indices in a very simple manner. If the inositol synthesis of these yeasts is too slow for rapid growth they then fall into a 123, 1234, or 1236 classification. Strain *alpinus* which has a B.N. 23 and the growth of which is inhibited by B<sub>1</sub> is typical of some other strains of *cerevisiae* yeasts.

Twenty-three strains representing 18 species of osmophilic *Zygosaccharomyces* were classified by Lochhead and Landerkin (7) into 3 groups headed A, B and C, according to their growth requirements. The basal medium contained ammonium sulfate as the nitrogen source, and 40% glucose in addition to 5 growth factors (nicotinic acid not used). Assigning bios numbers to the Lochhead and Landerkin data by the method herein proposed emphasized the similarity of the yeasts within each of their groupings which were 3 predominant types, i.e.,

B.N. 3, 23, and 123. Our collection contains 3 *Zygosaccharomyces* species (Table V) which have bios numbers of 7, 23, and 37, respectively. Thus, the feasibility of subclassifying *Zygosaccharomyces yeasts* into at least 6 types according to bios requirements is demonstrated.

Through the kindness of Prof. Paul R. Burkholder, we received representative species of the yeast cultures used in his studies of yeast growth factors. In 6 of the cultures, there was a lack of complete agreement between our results and those of Burkholder. The discrepancy in the case of *S. chodati* is explained by the fact that the Burkholder method of testing did not include simultaneous omission of B<sub>1</sub> and B<sub>6</sub>. The results of Burkholder *et al.* (5) show *S. chodati* to be a 235 yeast, whereas by our designation it is a 23 (B<sub>1</sub> inhibits growth) yeast. It is pertinent to note that without simultaneous omission of B<sub>1</sub> and B<sub>6</sub>, an important subspecies of *S. cerevisiae Hansen* would not have been discovered (1, 2). Lack of agreement upon bios requirements of some of the other Burkholder cultures may be due to differences in the media, and in the kind and amount of inoculum, as well as to the fact that in our tests the cultures were shaken during the propagation period. It should be stressed that the proposed use of yeast growth factors as a tool in taxonomy requires further research along collaborative lines.

The data in Table VI agree with the findings of Rogosa (11) in that all lactose-fermenting yeasts require an exogenous source of nicotinic acid for growth. However, in the same paper, Rogosa's general claim that "yeasts which do not ferment lactose do not require an exogenous source of nicotinic acid for growth" is not substantiated. Seven non-lactose-fermenting yeasts were found to require nicotinic acid for growth (Table VI). Rogosa's statement applies to the species of *S. cerevisiae Hansen* which he used as a basis for his conclusion, but it is not true of all yeasts and should be modified to that extent.

It would seem apropos to include in the name of each yeast the abbreviations of the sugars it ferments in addition to its bios number as proposed. The following 7 sugars would be involved, and where possible the first letter of each could be used as indicated: Dextrose (levulose, mannose), Galactose, Sucrose, Maltose,  $\frac{1}{3}$  raffinose, Raffinose completely, and Lactose. For example, an *S. cerevisiae Hansen* yeast requiring pantothenic acid and biotin as growth factors would have this notation, *S. cerevisiae Hansen* B.N. 23, D G S M r. A system of classifying yeast cultures on the basis of bios requirements is thus

suggested as a complement to the present classification methods which do not permit sufficient subdivision of species. This system should be an aid to the identification of species and genera as well as subspecies.

#### ACKNOWLEDGMENT

We wish to express our appreciation to Mr. Robert Fisher, Miss Jean Glasser, Miss Doris Knappe, Mr. Seymour Pomper, and Mr. Freeman R. Swift for their valuable assistance in this investigation.

#### SUMMARY

A simplified method for classifying yeasts according to their bios requirements has been presented. The basal medium contains casein hydrolyzate as a nitrogen source, and growth factors, inositol, calcium pantothenate, biotin, thiamine, pyridoxine, and nicotinic acid. A bios number (B.N.) assigned to each type indicates the factor or factors which, when individually omitted from the medium containing the other factors, will not permit full growth of the culture.

Strains, varieties or races of the species *Saccharomyces cerevisiae* Hansen and *Saccharomyces carlsbergensis* were subclassified into 13 and 6 types, respectively. Growth requirements were also determined and bios numbers assigned to some of the remaining species of the genus *Saccharomyces*. Eight types belonging to the genus *Torula* were determined. A few yeasts of other genera were also classified. Seven nicotinic acid-requiring yeasts were observed to be incapable of fermenting lactose.

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# The Proteins of Green Leaves. I. Isolation, Enzymatic Properties and Auxin Content of Spinach Cytoplasmic Proteins<sup>1</sup>

S. G. Wildman and James Bonner

*From the William G. Kerckhoff Laboratories of the Biological Sciences,  
California Institute of Technology, Pasadena 4*

Received April 28, 1947

## INTRODUCTION

The manner in which auxin<sup>2</sup>, the plant growth substance, exerts its effect in bringing about growth responses has been a subject of inquiry since the beginning of auxin work. It is now well known that a wide variety of growth responses can be elicited by auxin application, the response depending upon the nature and status of the plant tissue involved, and many workers have come to believe that auxin itself is associated with a general, basic cellular process. Because exceedingly minute amounts of auxin are sufficient to produce a growth response, and since the curve relating concentration of auxin applied to the amount of curvature produced in the *Avena* test bears close resemblance to the type of curve found for the combination of coenzyme with protein, it is probable that auxin may participate in an enzymatic reaction. As a result of this idea, numerous investigations have sought to show *in vitro* effects of auxin on enzyme systems (2, 16). No effects have yet been found, however, which suggest a clear relationship between auxin and any physiological enzymatic process.

It is now established for some plant tissues that most of the auxin is bound to cellular constituents. For example, Thimann and Skoog (18) have shown that auxin is released from *Lemna* tissue by proteolytic enzymes. In the spinach leaf, auxin is

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<sup>1</sup>Supported by a grant from the American Cancer Society.

<sup>2</sup>For convenience, the term auxin is herein limited to those chemical compounds which produce curvature in the *Avena* coleoptile. For other aspects of this problem, reference may be made to van Overbeek's recent and comprehensive review on plant growth regulators (16).



bound to proteins from which it can be released by proteolytic enzymes or by alkaline hydrolysis (23, 9). It is evident that these results lend credence to the view expressed above that auxin may participate in growth as a necessary constituent of an enzyme.

In the present work, a different approach to the study of auxin action has been undertaken. Experiments were performed with the aim of separating and characterizing the protein to which auxin is bound, and with the further aim of establishing the enzymatic properties of this protein. Spinach leaves were chosen as a source of material, partly because of the background of information concerning the auxin-protein of this plant, and mainly because large amounts of living plants are available the year-around.

While the primary objective of this work has been to elucidate the nature of the auxin-protein in spinach leaves, it has also been found necessary to enlarge upon existing methods (5, 10, 15) for the separation of the protoplasmic constituents of leaves and to develop methods for the separation of the cytoplasmic proteins.

Vickery (21), in a recent summary of the available information on the proteins of plants, has called attention to the need for using the more modern techniques of protein and enzyme chemistry before more progress can be accomplished in the field of leaf proteins. By using methods different from those used by earlier workers, we have found that the cytoplasmic proteins of spinach leaves can be separated into two distinct fractions, one of which constitutes about 75–80% of the total cytoplasmic proteins and appears to be electrophoretically homogeneous. The second, smaller fraction consists of a mixture of proteins. Each fraction of spinach protoplasm has been examined from the standpoint of bound auxin and enzymatic activity. As evidence for homogeneity or inhomogeneity of the protein preparations, extensive use has been made throughout this study of the Tiselius electrophoresis apparatus as a key analytical tool. Since the enzymatic properties of the proteins were to be investigated, it has been a routine practice to carry out all steps in the fractionation of leaf protoplasm as rapidly as possible and to maintain temperatures of 1°C., or lower, in order to prepare the proteins with as little denaturation as possible.

### *Structure of the Spinach Leaf Cell*

A typical, mature spinach leaf cell, Fig. 1, consists of a thin, peripheral layer of protoplasm surrounded on the exterior by a rather heavy cell wall and enclosing a large vacuole. Distributed uniformly throughout the protoplasm and embedded in the cytoplasm are the chloroplasts, bodies about 5–10  $\mu$  in diameter which contain all of the photosynthetic pigments. The nucleus represents an extremely small portion of the total protoplasm in a mature leaf cell, and, in the protoplasmic fractionation experiments to be described below, it has not been possible to follow the fate of the nuclear material, although it is suspected that the nuclear substance probably appears as a slight contaminant of the chloroplastic fraction.

It should be noted that protoplasm in an undisturbed leaf cell has a somewhat jellied appearance. Furthermore, the concentration of proteins in protoplasm must be relatively high, perhaps 20% or more, since only a very small fraction of the total volume of a mature leaf cell is actually occupied by protoplasm, and the vacuole is known to be nearly devoid of protein-like substances (5). However, the following terminology is used for convenience in this study: *whole protoplasm* = chloroplasts + cytoplasm + vacuole; *whole cytoplasm* = cytoplasm + vacuole.

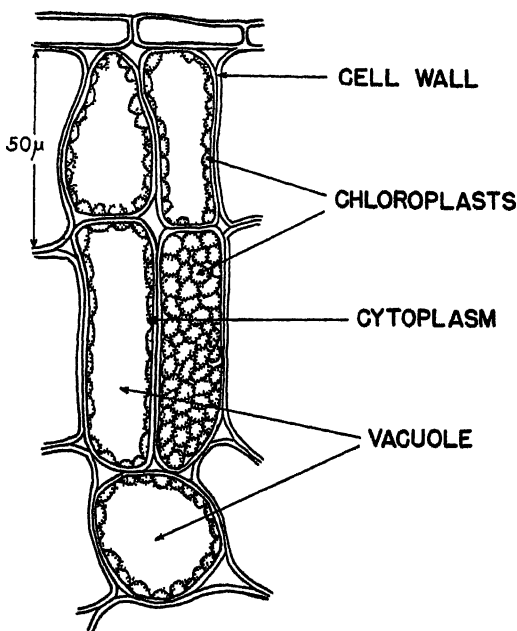


FIG. 1. Histological section through a mature spinach leaf showing palisade and spongy parenchyma cells. Cell to right with numerous chloroplasts is a top view of an intact cell. All other cells have been cut through and illustrate the peripheral distribution of leaf protoplasm enclosed by the cell wall and surrounding a large vacuole.

In a study of this kind, the cell wall must be broken in some manner and the protoplasmic contents dispersed into solution. Unfortunately, a very great change in the physical state of the protoplasm must accompany this initial step since the protoplasm immediately undergoes a relatively large dilution by the fluid contained in the vacuole. While there is little that can be done to overcome this difficulty, every effort has been made to prevent further dilution of the proteins of protoplasm during subsequent manipulations of the leaf material.

## METHODS AND MATERIALS

*Plant Material.* Spinach was obtained fresh from the field. The plants were removed from the soil in late afternoon with roots intact and placed upright in shallow pans of water. They were stored overnight in a cold room and used the following morning. The leaves used in the following experiments were in all stages of expansion, with the mature greatly predominant over the young and intermediate stages of growth. Before washing, the roots and petioles were removed and representative samples taken from nearly every leaf by means of leaf punches made with a sharp cork borer. The discs were weighed, thoroughly washed, rapidly dried at 80°C. in a forced-draft oven, finely ground and used for dry weight and nitrogen determinations. The remainder of the leaf tissue was weighed, washed in running water, drained, and cut into narrow strips for convenient handling. All subsequent operations were performed in a cold room maintained at 0–4°C.

*Auxin Determinations.* The standard *Avena* method as described by Went and Thimann (22) was used for auxin measurements and all values were demonstrated to be within the proportionality range of curvature *vs.* auxin concentration. Sensitivity of the test plants was determined for each experiment with crystalline indoleacetic acid. Results are expressed as curvatures and computed in terms of indoleacetic acid. Unknown solutions to be tested for auxin activity were acidified to pH 3 (glass electrode), extracted with recently distilled, peroxide-free ether and treated in the manner described by Wildman and Gordon (23).

*Electrophoresis Measurements.* Proteins were examined in a modified (20) Tiselius electrophoresis apparatus utilizing the Longworth scanning device. Conventional, double-length analytical cells were used. Before analysis, the proteins were dialyzed for 18 hours or more against 0.1 ionic strength buffers at 1°C., and then allowed to migrate in the same buffer generally for 3.5 hours with a current of 15 milliamperes. In order to obtain optimum separation, 1–2% protein solutions were chosen for analysis. Because of the color contained in most of the preparations, infra-red plates were used in photographing the scanning patterns.

*Dialysis.* The protein to be dialyzed was divided into 20 ml. portions, placed in 30 mm. Visking casings and the tied bags slowly rotated mechanically against large volumes of the dialyzing medium.

*Centrifuge.* For low speeds, a Servall SP type centrifuge was used. For high speeds, a large capacity centrifuge (320 ml.) equipped with a cooling coil and a vacuum jacket surrounding the head was available. With this apparatus, speeds as high as 20,000 r.p.m. (35,000 g) could be maintained for periods of 1 hour or more without encountering serious heating effects.

*Saturated  $(\text{NH}_4)_2\text{SO}_4$ .* A solution was saturated with the salt at 50°C., allowed to cool and crystallize at room temperature, the supernatant liquid filtered, the pH of the solution adjusted to 7.0 with concentrated  $\text{NH}_4\text{OH}$ , set aside in the cold room to allow further crystallization, and filtered before use. All saturation values refer to 71 g. of  $(\text{NH}_4)_2\text{SO}_4$  dissolved in 100 g. of water at 4°C.

*Protein Determinations.* The amount of protein in a solution was determined by either weighing a 0.50 ml. sample of thoroughly dialyzed material dried to constant weight at 90°C., or by precipitation of the protein with 1 ml. of 8% trichloroacetic

acid, centrifuging and washing the precipitate twice with distilled water, and weighing the precipitate after drying to constant weight at 90°C. The same values for protein concentration were found by either method.

*Separation of Cell Walls and Protoplasmic Juice.* Sliced leaves were ground with a minimum amount of water in a Stevens Blender for about one minute, the speed of the blender being controlled by a variable transformer so as to make possible the formation of a very viscous slurry. The slurry was then passed once through a Charlotte Colloid mill set for  $\frac{1}{2}$  maximum fineness of grinding. The blender pretreatment was necessary to prevent large leaf particles from clogging the colloid mill. The concentrated juice was separated from cell walls and unbroken cells by centrifuging the slurry in a small basket centrifuge lined with "sharkskin" filter paper which has pores large enough to prevent the passage of cell walls and intact cells, but which permits all of the dispersed protoplasm to pass through. The centrifuged juice was returned to the blender, more leaves added until a thick slurry was again formed, the slurry passed through the colloid mill and centrifuged. The process was repeated until all of the leaves to be used were extracted. The juice was then dried in the frozen state in a lyophil apparatus of the type described by Campbell and Pressman (4). Such dry material is designated *whole protoplasm*.

Usually, 1 kg. of leaves could be dispersed into approximately 500 ml. of liquid. By microscopic examination of the slurry before centrifuging, it was estimated that more than 50% of the leaf cells were ruptured and the protoplasmic contents dispersed by this treatment. The yield data in Table I confirm this estimate. The dry weight and nitrogen content of a portion of leaves removed with a cork borer was determined and the remainder of the leaf tissue was extracted in the manner described. The residue remaining on the filter paper after centrifuging was dried, weighed and analyzed for nitrogen. The total amount of lyophilized solids was weighed. With this information, it was found that from 77 to 91% of the original total amount of leaf solids was recovered either in the residue or as dispersed protoplasm. When the weight of the extracted protoplasm was compared to the weight of the starting material, it was found that from 38 to 47% of the total dry weight of the leaves had been dispersed into solution by this method of grinding. However, it might be expected that an appreciable part of the dry weight of spinach leaves would consist of water-insoluble cellulose, and on this account, a better index of the extent of extraction is based upon the amount of total nitrogen dispersed into solution. In the last column of Table I, the grams of nitrogen remaining in the residue after extraction have been divided by the grams of nitrogen in the original sample and results expressed as *per cent* of total nitrogen extracted. It will be seen that in the first three samples, 63-68% of the

TABLE I

*Extraction of Water-Soluble Constituents of Fresh Spinach Leaves*

Sample No.	Fresh wt. of Leaves extracted	Calculated dry weight	Per cent N in dry weight	Weight of whole protoplasm extracted	Residue		Dry wt. recovered	Per cent Recovery	Per cent Dry wt. extracted	Per cent Total N extracted
					Dry wt.	N Per cent				
1	g.	g.	g.	g.	g.	g.	g.			
1	1002	120	5.14	46	47	4.48	93	77	38.3(49.4 <sup>1</sup> )	66.0
2	1098	133	5.17	57	51	4.22	108	81	42.8(52.7 <sup>1</sup> )	68.7
3	1146	123	4.70	58	51	4.13	109	89	47.2(53.2 <sup>4</sup> )	63.5
4	500	51	5.28	24	23	3.24	46	91	47.1(52.2 <sup>2</sup> )	72.0

\* Figures within parenthesis based on actual amount of solids recovered rather than calculated amounts

total leaf nitrogen was extracted while, with the fourth sample, more than 70% of the original nitrogen was dispersed into solution. The nitrogen values compare very favorably with the microscopical estimation of the degree of rupturing.

By combining blending with the colloid mill treatment, it has been possible to obtain a much more concentrated protoplasmic juice than by blending alone. Compared to the colloid mill treatment, blending alone has two serious disadvantages: (1) as the volume of slurry increases, poorer mixing occurs so that not all of the sample in the blender is in contact with the blades for an equal length of time. With the colloid mill, all of the sample must pass through and hence, the sample is equally and thoroughly ground. (2) Rapid heating occurs in blending which is difficult to control even with a dry ice jacket surrounding the blender. To insure that 50% or more of the cells are broken, 5 minutes blending is a minimum requirement. During this period of time, a temperature rise of 10°C., or more, is not uncommon. With the colloid mill, heating effects are reduced to a minimum. Circulating ice-water surrounds the rotor and stator and the blended slurry passes through the mill very quickly with the result that the juice is not heated more than 2-3 degrees.

While it is possible to extract conveniently up to 75% of the total leaf nitrogen by this method, the question may be raised as to whether the nitrogen remaining in the residue after centrifuging represents unextracted protein which is insoluble in the protoplasmic juice. Chibnall (5) has emphasized that there is no reason to suspect any difference between the extractable and nonextractable cell constituents in his extraction procedure. He felt that the amount of protoplasm extracted was directly proportional to the number of leaf cells broken in the grinding treatment. The following experiment appears to strengthen this contention.

Leaves were treated in the above manner and the centrifuged semi-dry residues saved. Water was added in sufficient amounts to form a thick slurry and the residues reground through the colloid mill set for maximum fineness of grinding. The juice was

collected by centrifuging, and lyophilized. Water was again added to the centrifuged residue and the process repeated twice more. After each centrifuging, a small portion of the residue was dried and analyzed for total nitrogen and soluble nitrogen. Similarly, the nitrogen content of the lyophilized juice was determined after each extraction. After the final extraction, it was estimated by microscopical examination that 90% of the cells were broken. According to the data in Table II, both the soluble and total nitrogen content of the residue decreased with each successive extraction while the nitrogen content of the extracted lyophilized whole protoplasm remained the same. Since the nitrogen values for the protoplasmic juice remained constant, it seems doubtful if a different species of protein (or nitrogen-containing material) is extracted only by prolonged grinding. It is probable, as pointed out by Chibnall, that the nitrogen extracted by this method is fully representative of all of the total leaf nitrogen.

TABLE II

*The Effect of Long-Continued Grinding on the Nitrogen Content of the Extracted Protoplasm and the Insoluble Residue of Spinach Leaves*

No. of times extracted	Protoplasm per cent N	Residue	
		Per cent Total N	Per cent Soluble N
0		5.12	0.99
1	6.04	3.48	0.70
2	6.02	—	—
3	6.04	2.22	0.28

*Fractionation of Spinach Whole Protoplasm.* Microscopic examination of whole protoplasmic juice freed from cell wall fragments and unbroken cells, showed that the colloid mill treatment had caused complete disruption of the chloroplasts into much smaller particles or grana<sup>2</sup> (see below). Two methods have been used to separate chloroplastic material from other protoplasmic constituents.

Grana can be completely deposited by centrifuging a concentrated solution of whole protoplasm at 20,000 r.p.m. for one hour. Although all microscopically visible

<sup>2</sup> It was found that intact chloroplasts could be prepared from spinach leaves if the leaves were blended at full speed for not more than 30 seconds in the presence of an isotonic glucose solution, a slight modification of Granick's method (10). Sharkskin filter paper has pores large enough to permit the passage of whole chloroplasts through the paper. However, even with this extremely short period of blending, some of the chloroplasts were dispersed into grana; with longer periods, very few intact chloroplasts remained. Whole chloroplasts were readily separated from grana and cytoplasm by low speed centrifuging or allowing them to settle out overnight. The yields, of course, were low, since only about 20% of the leaf cells were broken by this treatment.

particles are thrown down by this treatment, the clear supernatant liquid still retains a slightly greenish cast suggesting that some of the grana are dispersed into submicroscopic particles. The second method combines precipitation of the grana with ammonium sulfate followed by low speed centrifuging. The first method has been used to prepare chloroplastic material and whole cytoplasm for use in nitrogen analysis and enzyme studies while the second has been used as a convenient method for removing grana before separating the cytoplasmic proteins.

*Structure of the Spinach Chloroplast.* Most investigators (see Rabinowitch (17)) now agree that chloroplasts consist of pigmented, rather symmetrical particles, or "grana," embedded in a nearly colorless matrix or "stroma." Just recently, Granick (11) has examined spinach chloroplasts under the electron microscope and elegantly confirmed the existence of grana. Such particles have a maximum dimension of about  $1.0 \mu$ . Apparently, the photosynthetic pigments are completely confined to the grana, but very little is known concerning the nature of stroma.

As previously mentioned, the colloid mill treatment causes complete disruption of spinach chloroplasts into grana. For this reason, mention of chloroplastic material in this investigation refers to pigmented grana preparations. Nothing can be said with certainty of the fate of stroma in the separation of leaf protoplasm. It is presumed that the stroma, if soluble, appears in the cytoplasmic fractions.

This matter is presented at this point in order that there will be no misinterpretation of the enzymatic studies to follow. For example, it has been variously reported that catalase is concentrated in the chloroplasts. In the present investigation, it will be shown that catalase is found in spinach cytoplasm. However, it is not possible to categorically state that catalase is not found in chloroplasts because it is possible that catalase is a constituent of stroma and that stroma appears in the cytoplasmic fraction. On the other hand, it is fairly certain that catalase is not associated with the *pigmented* particles of chloroplasts.

An attempt was made to isolate stroma. Intact spinach chloroplasts were prepared, washed several times with an isotonic glucose solution, and the intact chloroplasts then completely disrupted to grana by grinding the preparation in the colloid mill in the presence of distilled water. The grana were completely removed by centrifuging at 20,000 r.p.m. The clear supernatant was concentrated by lyophilization leaving an extremely small amount of material. The experiments were not continued because it was felt that spinach chloroplasts are not optimum material for a study of stroma preparation. It is very difficult to prevent the disruption of chloroplasts even with mild grinding operations in the presence of isotonic solutions, thus preventing ade-

quate washing of the intact chloroplasts to completely free them of cytoplasm. It would probably be better to use a plant such as tea or tobacco where the disruption of intact chloroplasts can be performed only by very drastic grinding procedures.

*Separation and Purification of Grana by Centrifuging.* Since the amounts of grana and cytoplasmic proteins vary somewhat as a reflection of the metabolic condition of the leaves at the time of extraction, a typical experiment will be described. Thirty g. of whole protoplasm were slowly redispersed with rapid stirring into 200 ml. ( $\frac{1}{2}$  the volume of solution from which the preparation was originally dried) of ice-cold, distilled water and centrifuged 1 hour at 20,000 r.p.m. All microscopically visible particles were deposited in this time, although the clear supernatant liquid had a slightly greenish cast.<sup>4</sup> As indicated in Table III, 4.86 g. of cytoplasmic proteins were

TABLE III

*The Amount of Cytoplasmic Protein Removed by Successive Extractions with Water of 30 g. of Whole Protoplasm.*

Protein determined by trichloroacetic acid precipitation

	Total volume of supernatant liquid ml.	Total g. of cytoplasmic protein extracted
First extraction	223	4.86
Second extraction	97	0.66
Third extraction	100	0.32
Total Protein		5.84

extracted in the supernatant liquid. After decanting, the precipitated grana were re-extracted by blending for 2-3 minutes with 100 ml. of water to insure thorough dispersion of the particles. The suspension was centrifuged for 1 hour at 16,000 r.p.m. The clear supernatant contained 0.66 g. of protein and was free of green color. A third extraction yielded 0.32 g. of protein. It is, therefore, evident that more than three extractions are required to completely free the grana from water-soluble proteins. The final grana precipitate was dispersed in 50 ml. of water and lyophilized. The grana weighed 8.1 g. and represented 32.8% of the whole protoplasm and contained 7.34% N. Cytoplasmic proteins (5.84 g.) were extracted, equal to 19.4% of the whole protoplasm. Hence, 52.2% of whole protoplasm can be considered protein in nature.

The clear supernatants were combined (420 ml.) and concentrated to approximately 200 ml. by distributing the solution in dialysis sacks and blowing air on them with a fan for 24 hours at 1°C. The bags were then tied to prevent dilution and dialyzed against 2 l. of distilled water for 24 hours. Dialysis caused precipitation of the remaining traces (100 mg.) of chlorophyll-protein, which was removed by centrifuging the solution for 1 hour at 4000 r.p.m. The remaining solution of cytoplasmic proteins was clear and free from green color.

<sup>4</sup> At the bottom of the grana precipitate, a white layer of material of unknown constitution was deposited. It is possible to separate this material from grana by differential centrifugation. The amount of ash remaining after ignition led to the conclusion that the material is probably at least partly inorganic in nature.



It is of interest to examine the distribution of nitrogen in the spinach leaf. Using the data for sample 3, Table I, 63.5% of the total leaf nitrogen was extracted as whole protoplasm which contained 32.8% grana and 19.4% cytoplasmic proteins. If it is assumed that spinach cell walls contain little or no nitrogen<sup>5</sup> and that, with exhaustive grinding, nearly all of the nitrogen would have been extracted, then it can be estimated that 81.6 g. of protoplasm might have been extracted from 1 kg. of fresh leaves, or, of the 107 g. of dry matter found for this batch of leaves, the difference, or 2.5% of the fresh weight, can be considered as cell walls.

TABLE IV

*Approximate Distribution of the Cellular Constituents of the Spinach Leaf*  
(See text for method of calculation)

Part of leaf cell	Per cent Dry wt	Per cent Fresh wt.	Per cent Total leaf N
Cell walls	23.8	2.5	Presumably low
Protoplasm	76.2	8.2	Presumably 100
1. Chloroplasts	26.8	2.7	37.9
2. Cytoplasmic proteins	15.8	1.9	39.3
a. Fraction I	9.2	0.98	—
b. Fraction II	3.1	0.32	—
3. Low molecular weight, non-protein, water-soluble substances	33.6	3.6	22.8

Similarly, in 81.6 g. of protoplasm, 26.8 g., or 2.7% of the fresh weight of the spinach leaf, is made up of grana. Of the total leaf nitrogen, 37.9% is found in this fraction, a value in good agreement with the 30–40% nitrogen found for tobacco and tomato leaves by Granick (10) and for grass leaves by Hanson (13). One and nine-tenths per cent of the fresh weight consists of cytoplasmic proteins, and 39.3% of the total nitrogen is found in this fraction. Thus, 77.2% of the total leaf nitrogen appears to be mainly of a protein nature. The data in Table IV, showing the approximate distribution of the cellular components of the spinach leaf, have been calculated by this method.

<sup>5</sup> Crook (6), in a study of extraction methods, has found that 95% of the total protein in tobacco leaves can be extracted by suitable methods. Analysis of the insoluble residue showed it to consist mostly of carbohydrate and about 1% N.

*Electrophoretic Analysis of Whole Cytoplasm.* In view of the ease with which the different blood-serum proteins can be identified as a mixture of several components by using the Tiselius moving-boundary apparatus, dialyzed spinach cytoplasm was examined to determine whether the proteins could be resolved into separate components by migration in an electrical field.

Grana were removed from whole protoplasm by centrifuging, and the supernatant dialyzed to remove traces of green color by precipitation together with the low molecular weight, dialyzable constituents. One and five-tenths *per cent* solutions of cytoplasmic proteins were finally dialyzed for 24 hours against phosphate buffers of 0.1 ionic strength and different pH values, and then allowed to migrate in a Tiselius apparatus for 3.5 hours in the same buffer. No separation occurred in either the ascending or descending boundaries at pH 6.8, 7.2, or 8.0. In fact, the single, prominent peak became even narrower with increasing alkalinity of the buffer. At pH 6.4, however, a slight differentiation emerged as evidenced by broadening of the peak at the base, together with the appearance of a small hump in the ascending boundary

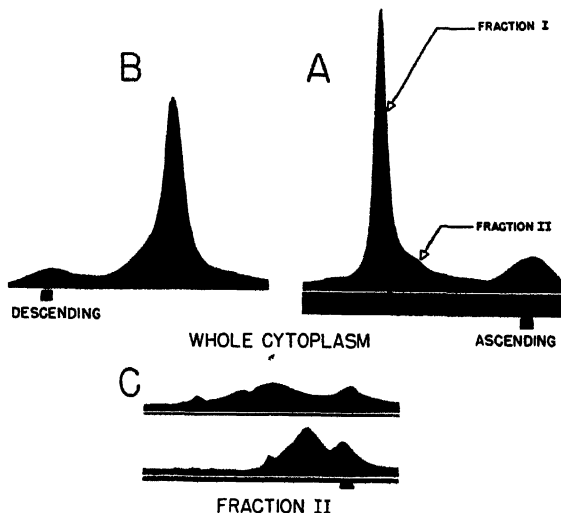


FIG. 2. Longsworth scanning patterns of spinach leaf proteins after 3.5 hours migration in a Tiselius electrophoresis apparatus. A, ascending boundary of whole cytoplasmic proteins representing the maximum separation yet observed. B, descending boundary of the same preparation. C, electrophoretic character of Fraction II after complete separation from Fraction I. Several components are apparent. Top, after 3.5 hours migration; bottom, 2 hours. All samples were run in phosphate buffer, pH 6.4, 0.1 ionic strength; 15 milliamperes current. Starting boundaries indicated immediately below the scanning patterns.

representing a component migrating at a rate slightly slower than the main component, as shown in Fig. 2. Because of partial irreversible precipitation at the isoelectric point, it was not possible to examine the electrophoretic behavior of the cytoplasmic proteins at a pH more acid than 6.2.

These results suggest that the cytoplasm consists of a mixture of proteins, but, on considering the areas occupied by the two components in the scanning patterns, it can be estimated that about 75% of the total proteins consists of a single, electrophoretically homogeneous protein. As will be described below, it is possible to separate the main protein from the less conspicuous smaller component by fractional precipitation with  $(\text{NH}_4)_2\text{SO}_4$ . The smaller component, when free of Fraction I, consists of a mixture of proteins as shown by the scanning pattern in Fig. 2.

Frampton and Takahashi (7) have examined the protein of healthy and virus-infected tobacco leaves. Ether-cytolyzed leaves were ground and extracted with buffer, centrifuged to remove debris, and dialyzed against buffer. The extracts were centrifuged for a few minutes at high speed to remove plastids, and the supernatant fluid examined in a Tiselius apparatus. Scanning patterns indicated the presence of two major and one minor protein components, representing what was considered three groups of proteins, none of which were resolved electrophoretically under the conditions used. The method of extraction precluded precise determinations of the concentrations of the several components.

There is a striking similarity between the migration characteristics of the main slower component in tobacco and spinach leaves. A third component has sometimes appeared in spinach cytoplasmic preparations which corresponds to component A of tobacco. However, the appearance of this component in spinach has always been associated with a slight green color in the solution being analyzed. When the green color is removed by dialysis and centrifuging, the extra peak also disappears from the scanning pattern.

*Fractionation of the Cytoplasmic Proteins.* To facilitate the fractionation of the cytoplasmic proteins without the inconvenience of high speed centrifugation, the following method was used to separate grana from cytoplasm. Fifteen g. of whole protoplasm were dispersed with rapid stirring into 100 ml. of an ice-cold solution of 0.25 saturated  $(\text{NH}_4)_2\text{SO}_4$  and the solution centrifuged one hour at 4,000 r.p.m. With this concentration of  $(\text{NH}_4)_2\text{SO}_4$ , the grana are effectively held out of solution but are sufficiently wetted so that they pack well into the bottom of a centrifuge tube. To the clear, cherry-colored, supernatant was added dropwise enough saturated  $(\text{NH}_4)_2\text{SO}_4$

solution to give 0.35 saturation, causing a heavy precipitate to form. The precipitate was removed by centrifuging for one hour at 4,000 r.p.m. After decanting (supernatant I), the precipitate was completely redissolved in 50 ml. of cold phosphate buffer, pH 6.8 (Fraction IA).

Supernatant I was distributed into four dialysis sacks and concentrated by fanning for 24 hours. Reduction in volume by one-third in the presence of  $(\text{NH}_4)_2\text{SO}_4$  produced a second precipitate which was removed by centrifuging one hour at 4,000 r.p.m. Supernatant II was decanted and the precipitate redissolved in 25 ml. of phosphate buffer (Fraction IB).

Supernatant II was further concentrated to one-half volume without producing more precipitate. The bags were then tied to prevent excessive dilution by osmosis and the solution dialyzed against distilled water until the bags were tight. The pressure was relieved by fanning, the solution dialyzed against distilled water, and the process repeated until the protein solution was free from salt. The solution was then concentrated to one-fourth the original volume, the bags tied to prevent dilution, and the solution dialyzed against 16 l. of running distilled water. A precipitate formed which was removed by centrifuging for one hour at 4,000 r.p.m. The precipitate was redissolved in 10 ml. of phosphate buffer (Fraction IC) and the supernatant electro-dialyzed against 10 l. of running distilled water to remove final traces of salt. This treatment produced a small precipitate which was collected by centrifuging and dissolved in 10 ml. of phosphate buffer (Fraction ID). The supernatant after electro-dialysis and centrifuging was perfectly clear, bluish-green in color, and was designated *Fraction II*. Fig. 3 is a simplified, schematic representation of the methods used in fractionating spinach protoplasm.

Fractions IA, IB, IC, and ID were analyzed in the Tiselius apparatus and were found to have almost identical mobilities with no apparent difference in the shape of the patterns. They were, therefore, combined and used for further purification studies. The combined proteins are hereafter designated *Fraction I*.

*Bound Auxin Content of Spinach Proteins.* After separation of the grana and fractionation of the cytoplasmic proteins, the various protoplasmic fractions were analyzed for bound auxin.

Twenty mg. samples of grana, Fractions IA, IB, IC, and ID, and Fraction II were separately hydrolyzed with 5 ml. of 0.1 *N* NaOH for 8 hours at 100°C., and the released auxin extracted with ether after acidification of the hydrolyzate to pH 3.0. The grana sample had been prepared by centrifuging and had been extracted three times with water. As the data in Table VI show, no auxin was released from the grana or Fraction II by alkaline hydrolysis, but substantial and nearly identical amounts were released by the same treatment from Fractions IA, IB, IC, and ID, fractions previously shown to have the same electrophoretic migration characteristics. The experiment was repeated using trypsin as the hydrolyzing agent. Sixty mg. samples of protein were incubated 48 hours with 10 mg. of Armour's trypsin at 37°C. in 5 ml. phosphate buffer, pH 7.4. No auxin was released from Fraction II, a small amount from the grana, and much larger amounts from Fractions IA and IB. Not enough

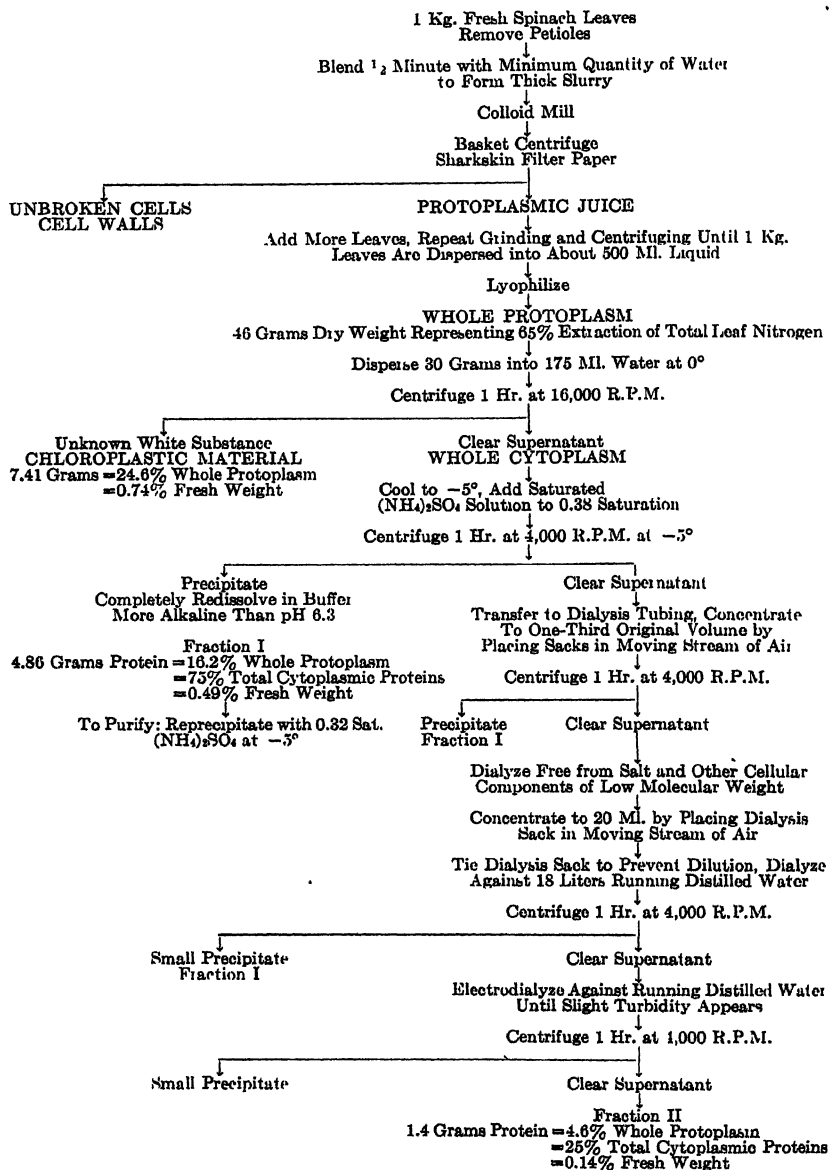


FIG. 3. A schematic summary of the method used for separating spinach protoplasm.

TABLE V

*The Amount of Cytoplasmic Proteins Precipitated by Various Treatments*  
(See text for details)

Protein fraction	Method of preparation	Per cent Total cytoplasmic proteins
IA	0.35 Sat. $(\text{NH}_4)_2\text{SO}_4$	50
IB	Concentration by fanning in presence of salt	15
IC	Water dialysis	5
ID	Electrodialysis	2
II	Supernatant after electro-dialysis	25

TABLE VI

*The Bound Auxin Content of Various Fractions of Spinach Leaf Protoplasm*

Protoplasmic constituent	Weight in mg.	Method of hydrolysis	Ml. of agent	Auxin curvature in degrees	Mg. IAA $\times 10^{-7}/10$ mg. protein
Grana	60	Trypsin	0.80	$15.8 \pm 0.9$	0.7
Grana	20	0.5 N NaOH	0.80	0	0
Fraction II	60	Trypsin	0.80	0	0
Fraction II	20	0.5 N NaOH	0.80	0	0
Fraction IA	60	Trypsin	3.20	$20.9 \pm 1.8$	4.0
Fraction IA	7.3	Pepsin + Trypsin	3.20	$2.9 \pm 0.6$	8.0
Fraction IA	10	0.5 N NaOH	6.40	$3.2 \pm 0.6$	8.3
Fraction IB	10	0.5 N NaOH	6.40	$2.8 \pm 0.8$	7.2
Fraction IC	10	0.5 N NaOH	6.40	$3.6 \pm 0.6$	9.3
Fraction ID	10	0.5 N NaOH	6.40	$3.1 \pm 0.8$	8.0

protein was available for enzymatic digestion of Fractions IC and ID. It should be noted that approximately 6 times as much auxin was released from Fractions IA and IB by treatment with hot alkali as with trypsin, but it has since been found that, when pepsin is added to the protein for 24 hours followed by trypsin, the yield of auxin is equal to the amount liberated by alkali.

Prolonged dialysis of Fraction IC and electrodialysis of Fraction ID did not decrease the bound auxin content of these proteins, a result in contrast to Gordon's (8) finding that electrodialysis of wheat proteins appeared to remove the auxin. Dialysis of the protein against cold, 5 N NaOH or treatment of the protein with cold, concentrated HCl failed to release the auxin.

Incubation of the auxin-protein at pH 10.5 for 48 hours, a treatment used by Haagen-Smit *et al.* (12) to release auxin from wheat seeds, failed to release auxin from Fraction I. Treatment with 0.1 N HCl in cold acetone was also ineffective. Thus, auxin appears to be firmly bound to the protein.

The reproducibility of these results for the bound auxin content of Fraction I should be emphasized. During the course of this investigation over a period of two years, some 25 batches of Fraction I have been prepared. Bound auxin has been found associated with this protein in every case and the amount of auxin released from equal weights of protein have been remarkably constant, notwithstanding the fact that the plants were growing at different seasons of the year and frequently under quite different cultural conditions.

*Enzymatic Activities of the Cytoplasmic Proteins.* It is evident that among the cytoplasmic proteins of spinach leaves must be included those proteins which constitute the soluble enzymes of the plant cell, and it has been established during the course of the present work that whole cytoplasm does, in fact, exhibit different types of enzymatic activities. Of the enzymes which have been studied, all but one are not found in Fraction I after purification. Some of the others have been identified in Fraction II and will be discussed below. Fraction I is associated with a phosphatase activity, as is shown in the following experiments.

Ten mg. samples of protein were incubated at 37°C. with 10 mg. of  $\beta$ -glycerophosphate in 6.0 ml. of 0.1 *M* KOH-maleate buffer at pH 6.0. One ml. aliquots of the enzymatic digest were withdrawn after 0, 15, 30 and 60 minutes time and added to 1.0 ml. of 8% trichloroacetic acid. The aliquot was diluted with water, 1.00 ml. of 2 *N*  $H_2SO_4$ , 0.50 ml. ammonium molybdate, and 0.50 ml. of Fiske-SubbaRow (6) reducing agent added and the solution made to 25 ml. volume. The solutions were filtered, the color allowed to develop for 15 minutes, and the amount of inorganic phosphorus determined by a photoelectric colorimeter. The data are shown in Table VII.

TABLE VII

*The Phosphatase Activity of Various Fractions of Spinach Cytoplasmic Proteins*

Enzyme	Preparation	Percentage of theoretical amount of P released from 10 mg. of $\beta$ -glycerophosphate in 15 minutes by 10 mg. of protein
Fraction IA	$(NH_4)_2SO_4$ (Heat denatured)	0
Fraction IA	$(NH_4)_2SO_4$ (Heat denatured)	19.8
Fraction IB	Concentrated by fanning in presence of salt	20.0
Fraction IC	$H_2O$ Dialysis	27.7
Fraction ID	Electrodialysis	16.5
Fraction II	Supernatant after electrodialysis	11.2

Fractions IA and IB were equal in their ability to liberate inorganic phosphate from  $\beta$ -glycerophosphate and this property was lost when the protein was denatured by heating. Fraction ID, prepared by electrodialysis, was less active, while the protein prepared by dialysis alone, displayed the greatest activity. While Fraction II was active in liberating phosphate, it was, nevertheless, considerably less active than any preparation of Fraction I and was less than one-half as active as Fraction IC. It is evident that the proteins containing bound auxin have the highest phosphatase activity. Since it has been difficult to remove all traces of Fraction I from Fraction II it cannot be known with certainty whether the phosphatase activity of this fraction represents a different phosphatase or merely contamination by Fraction I.

*Reprecipitation, Electrophoretic Behavior, Auxin Content and Phosphatase Activity of Fraction I.* Since Fraction I was found to be the principal source of bound auxin in the spinach leaf, to have the exclusive enzymatic properties of a phosphatase, and to constitute such a strikingly large part (75%) of the total cytoplasmic proteins, it was of considerable interest to critically test the apparent homogeneity of this protein.

Saturated  $(\text{NH}_4)_2\text{SO}_4$  solution was added dropwise with rapid stirring to 4.86 g. of a new preparation of cytoplasmic proteins, prepared according to the above scheme, contained in 175 ml. of phosphate buffer, pH 6.8. The protein which precipitated at 0.35 saturation was removed by centrifuging and redissolved in about 45 ml. of phosphate buffer, pH 6.8 and made to 60 ml. volume in the same buffer. As Table VIII

TABLE VIII

*The Effect of Successive Precipitations of Fraction I with  $(\text{NH}_4)_2\text{SO}_4$  on the Bound-Auxin Content and Phosphatase Activity of the Protein*

Fraction	Preparation	Per cent Starting protein	Auxin content mg. IAA $\times 10^{-4}$ /10 mg protein	Phosphatase activity <sup>4</sup>
IE	Whole dialyzed cytoplasm	100	<3.0	19.8
IF	First precipitation with 0.35 $(\text{NH}_4)_2\text{SO}_4$	53.0	6.7	13.2
IG	Second precipitation with 0.33 sat. $(\text{NH}_4)_2\text{SO}_4$	37.8	6.7	7.9
IH	Third precipitation with 0.33 sat. $(\text{NH}_4)_2\text{SO}_4$	30.1	6.7	4.9

\* Percentage of theoretical amount of P released by 10 mg. of protein in 15 minutes at pH 6.0 from 10 mg.  $\beta$ -glycerophosphate.



indicates, 53% of the total cytoplasmic proteins precipitated with this concentration of salt. A 10 ml. aliquot of the protein was set aside (Fraction IE) and the protein reprecipitated with 0.33 saturated  $(\text{NH}_4)_2\text{SO}_4$ . Of the original starting protein (whole cytoplasmic proteins), 37.8% was recovered after removing the protein by centrifuging and redissolving it in phosphate buffer and making the volume again to 60 ml.

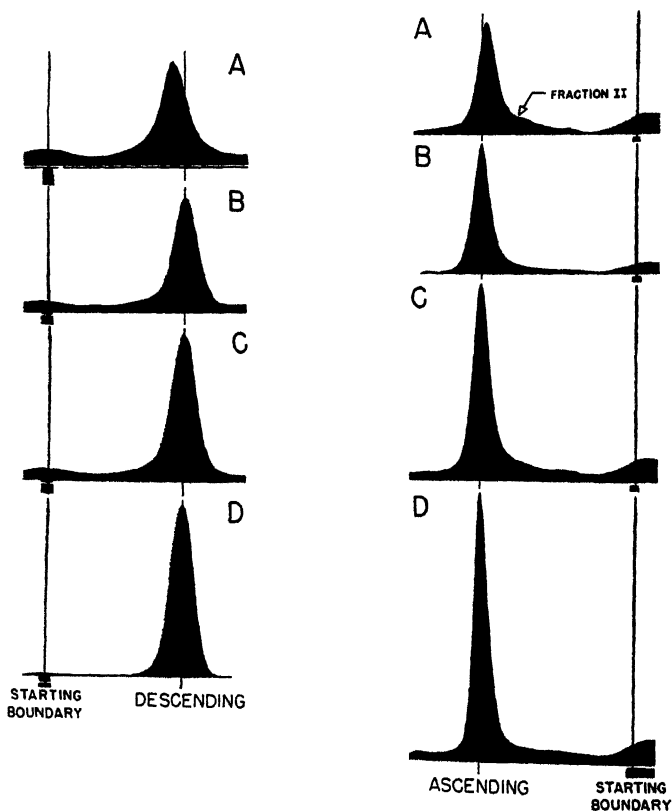


FIG. 1. Longworth scanning patterns of whole cytoplasmic proteins and Fraction I after successive precipitations with  $(\text{NH}_4)_2\text{SO}_4$ . A, whole cytoplasmic protein. B, Fraction IE; C, Fraction IF; D, Fraction IG. 3.5 hours migration in phosphate buffer, pH 6.5, 0.1 ionic strength, 15 milliamperes current.

Another 10 ml. aliquot was removed (Fraction IF). The protein was again precipitated and treated as before. Of the starting protein 30.1% was recovered (Fraction IG). The starting protein and the three fractions were dialyzed against 4 l. of the same buffer and used for auxin determinations, mobility studies, and phosphatase activity.

Electrophoresis analysis showed that while the starting protein contained about 25% Fraction II, the fractions prepared by successive precipitations with 0.33 saturated  $(\text{NH}_4)_2\text{SO}_4$  solution were identical in so far as the shape of the scanning patterns and the measured mobilities of the proteins can be compared. Fig 4. shows how well the boundaries of the three fractions superimpose after the proteins had migrated for 3.5 hours in an electrical field. Thus, three successive precipitations of the protein failed to reveal the presence of a significant electrophoretical impurity even though 70% of the original starting protein was discarded in the process of purification.

Similarly, auxin analysis of the individual protein fractions emphasized the apparent homogeneity of the fractions prepared by  $(\text{NH}_4)_2\text{SO}_4$  precipitation. Ten mg. samples of protein were hydrolyzed for 2 hours with 1 N NaOH in an autoclave at 15 lbs. pressure. With this weight of protein, not enough auxin was released from the unfractionated cytoplasmic proteins to produce curvature in *Avena* test plants (less than  $0.20 \times 10^{-5}$  mg. IAA/mg. of protein). However,  $0.67 \times 10^{-5}$  mg. of IAA/mg. of protein was obtained from the protein precipitated with 0.35 saturated  $(\text{NH}_4)_2\text{SO}_4$  and this amount of auxin was not increased or decreased by successive precipitations of the protein, as shown in Table VIII.

Phosphatase activity, on the other hand, decreased with each successive precipitation with about one-half the activity being lost after each precipitation. All steps in this experiment were performed at  $\pm^\circ\text{C}$ . The precipitation experiment was repeated using a new preparation of Fraction I made from the same batch of spinach leaves, but, to reduce denaturation as much as possible, all manipulations including centrifuging were carried out at  $-5^\circ\text{C}$ . instead of at  $\pm^\circ\text{C}$ . as in the previous experiment. The results of this experiment are compared to the previous experiment in Table IX. It is evident that the colder temperature appreciably reduced the loss in phosphatase activity experienced previously, even though the amount of protein discarded with each precipitation was about the same. The initial drop in activity attendant on the first precipitate may well represent another phosphatase present in Fraction II which is lost with the first precipitation. It is to be stressed again that, in view of the difficulty of completely freeing Fraction II of Fraction I, it has not yet been possible to decide with certainty whether Fraction II also contains phosphatase activity. In our opinion, the loss in activity resulting from subsequent precipitations

can probably be attributed to partial denaturation of the protein in the presence of high concentrations of  $(\text{NH}_4)_2\text{SO}_4$ . While the protein completely dissolves into the buffer after each of the described precipitations, it has been repeatedly observed that the time required for complete solution becomes progressively longer after each precipitation, indicating some change in the solubility characteristics of the protein.

TABLE IX

*Comparison Between the Phosphatase Activity of Fraction I after Successive Precipitations at Two Different Temperatures*

Number of precipitations	Per cent Original $\beta$ -glycerophosphatase activity			
	+4°C.		-5°C.	
	Per cent Protein recovered	Activity	Per cent Protein recovered	Activity
—	—	100*	—	100**
1	53	66.7	50	69.5
2	38	40.0	33	64.0
3	30	25.0	23	53.0
4	—	—	16	40.3

\* 24.6% of theoretical amount of organic P released by 10 mg. of Fraction I from 10 mg.  $\beta$ -glycerophosphate in 30 minutes at pH 6.0, 30°C.

\*\* 23.8% of theoretical amount of organic P released by 10 mg. of Fraction I from 10 mg.  $\beta$ -glycerophosphate in 30 minutes at pH 6.0, 30°C.

A fifth precipitation, in several instances, sufficiently denatured the protein so that only about one-half of the precipitate could be dissolved in buffer, and then only slowly and with difficulty.

*Further Studies on the Enzymatic Activities of Fraction I.* It was of interest to examine Fraction I more critically for possible types of enzymatic activity. The following enzymatic activities were sought and were specifically missing in Fraction I: polyphenol oxidase, catalase, peroxidase; malic, citric, isocitric, glutamic and alcohol dehydrogenases; aconitase, hexokinase, and tryptophan-converting enzyme (24). All of these activities can be readily demonstrated in whole cytoplasm and many have been found in Fraction II, although the long dialysis required in the preparation of Fraction II apparently inacti-

vates some types of enzymes. Spinach leaf breis and Fraction I appear not to contain the following enzymes: cytochrome oxidase, D-amino acid oxidase, phosphorylase, diastase, succinic dehydrogenase, and others. Only phosphatase activity has yet been shown to be associated with Fraction I.

Fig. 5 shows that optimum hydrolysis of  $\beta$ -glycerophosphate by Fraction I occurs at pH 5.25, which is very nearly the isoelectric point of the protein.

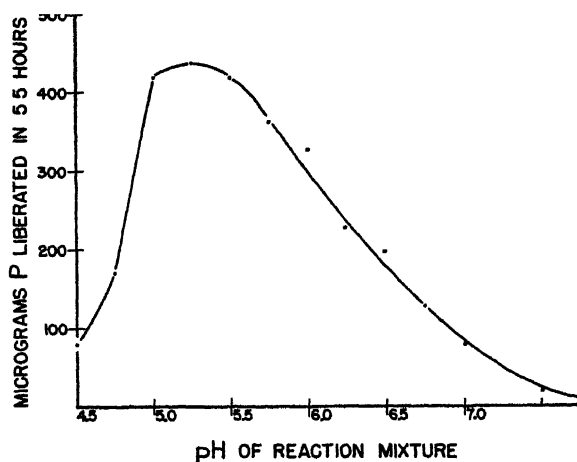


FIG. 5. The relationship between the phosphatase activity of Fraction I and the pH of the reaction mixture. Reaction mixture contained 5.0 ml. maleate buffer, 1.0 mg. Fraction I, 50 mg.  $\beta$ -glycerophosphate. Temperature 21°C.

TABLE X

*Effect of Added Sodium Fluoride on the Phosphatase Activity of Fraction I*

Reaction mixture contains per ml.: 2 mg. Fraction I, 10 mg. Na  $\beta$ -glycerophosphate. Maleate buffer pH 6.0

Na F concentration mg./ml.	$\gamma$ Inorganic P liberated in 135 minutes
0.0	128
0.1	56
1.0	31

Fig. 6 shows that saturation of the enzyme with substrate does not occur until relatively large amounts of  $\beta$ -glycerophosphate are added to the enzyme mixture.

The data in Table X show that small amounts of fluoride greatly inhibit the  $\beta$ -glycerophosphatase activity of Fraction I. One mg. of NaF/ml. resulted in 76% inhibition of the liberation of inorganic P

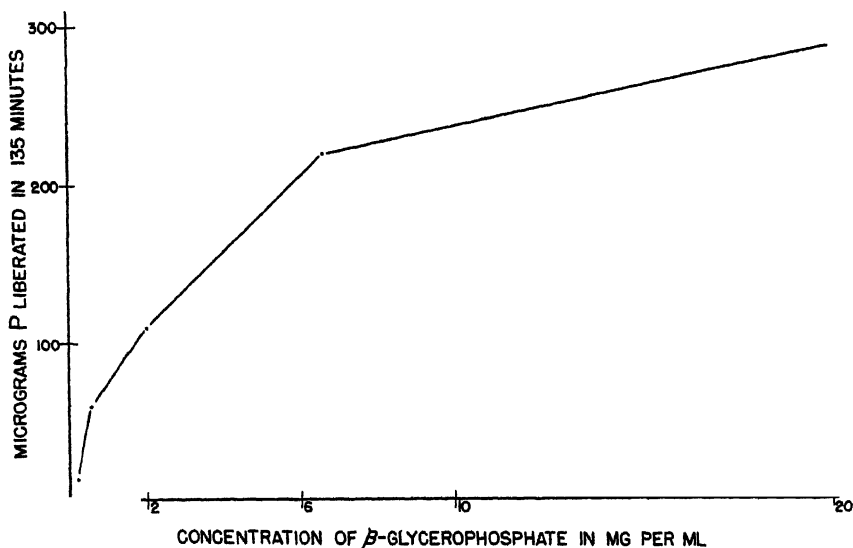


FIG. 6. The relation between the rate of phosphatase reaction of Fraction I and the concentration of substrate. Reaction mixture contained 5.0 ml. maleate buffer, pH 5.5, 1.0 mg. Fraction I and varying amounts of  $\beta$ -glycerophosphate. Temperature 21°C.

from  $\beta$ -glycerophosphate. The rate of reaction of the enzyme was not influenced by the addition of Mg either in the presence or in the absence of F. Addition of IAA was without any effect on  $\beta$ -glycerophosphatase activity when tested over a wide range of concentrations as shown in Table XI.

Listed in Table XII are the types of phosphorylated compounds which 3-times precipitated Fraction I can attack. In addition to  $\beta$ -gly-

TABLE XI

*Lack of Effect of Added Indoleacetate on the Phosphatase Activity of Fraction I*

Reaction mixture contains per ml.: 2 mg. Fraction I, 10 mg. Na  
 $\beta$ -glycerophosphate. Malcate buffer pH 6.0

Added indole- acetic acid mg./5 ml.	$\gamma$ Inorganic P liberated in 135 minutes
None	160
1 mg.	156
0.1	156
0.01	156
0.001	160

cerophosphate, the enzyme of Fraction I attacks fructose-1,6-diphosphate and phytic acid at a rapid rate. Glucose-1-phosphate and fructose-6-phosphate are hydrolyzed more slowly, while glucose-6-phosphate does not appear to be attacked at all.

It is an interesting fact that Fraction I is able to hydrolyze such high energy phosphate compounds as creatine phosphate and adenosine triphosphate. Since the hydrolysis of ATP under these conditions only leads to dissipation of the bond energy as heat, a few attempts were made to cause the transfer of phosphate from ATP to the guanido group of creatine, arginine, or glycocyamine. The results were either negative or equivocal. Similarly, it has not been possible to demonstrate the phosphorylation of fructose-6-phosphate in the presence of enzyme and ATP.

*Other Properties of Fraction I.* Data bearing on the chemical and physical properties associated with Fraction I will be the subject of a separate communication. It is of interest to note here that a sugar residue is found on hydrolysis of the protein, and that the nitrogen content of the protein, 12.7%, is lower than usual for most proteins. The purified protein is cherry colored in 1% solutions or greater, but shows no absorption maxima in the visible region of the spectrum as illustrated in Fig. 8. Fig. 7 shows that an absorption maximum occurs in the ultraviolet at 280  $m\mu$ . Fractions IA and IC (page 393) were compared in equal concentrations, 0.1 mg. protein/ml. phosphate buffer, and it is evident that the ultraviolet absorption spectra of the two fractions very closely superimpose. Because of the large absorption by

TABLE XII  
*Phosphorylated Compounds Attacked by Fraction I*

Substrate	Mg. substrate/ ml. reaction mixture	pH	Temperature	$\gamma$ of P liberated in 15 minutes by 10 mg. of Fraction I
			'C.	
$\beta$ -glycerophosphate <sup>d</sup>	1.0	6.0	30	36
Creatine phosphate <sup>d</sup>	11.0	6.9	35	38
Adenosine triphosphate <sup>e</sup>	3.6	6.0	30	26*
Fructose-1,6-diphosphate <sup>b</sup>	3.0	6.0	24	60
Phytic acid <sup>c</sup>	3.0	6.0	24	90
Fructose-6-phosphate <sup>d</sup>	3.0	6.0	24	18
Glucose-1-phosphate <sup>c</sup>	3.0	6.0	24	8
Glucose-6-phosphate <sup>c</sup>	3.0	6.0	24	0

\* 14% of terminal P. In one hour, the same preparation hydrolyzed 60% of the total 7-minute P of ATP.

<sup>d</sup> *Creatine P.* Calcium salt of Creatine P dissolved in enzymatic reaction mixture at 0°C. Aliquot withdrawn immediately after mixing and reaction stopped by 1.00 ml. 8% trichloroacetic acid. (All steps performed at 0°C. to prevent hydrolysis of labile phosphate.) Aliquot neutralized after removing precipitated protein by centrifuging.  $\text{CaCl}_2 \cdot \text{Ca}(\text{OH})_2$  reagent added to solution to precipitate inorganic P. Solution centrifuged to remove  $\text{Ca}_3(\text{PO}_4)_2$ . Remaining creatine P hydrolyzed at room temp. by adding 1 cc. 1 N  $\text{H}_2\text{SO}_4$  and waiting 15 minutes before adding Fiske-SubbaRow color developing reagents. Other aliquots removed after 30, 60 and 120 minutes at 35°C.

<sup>e</sup> *ATP.* Ba Salt of ATP dissolved in 1 N HCl at 0°C. Slight excess of  $\text{Na}_2\text{SO}_4$  added, and  $\text{BaSO}_4$  removed by centrifuging. ATP solution neutralized, added to buffered enzymatic reaction mixture at 0°C. Aliquot removed immediately after mixing and added to 1.00 ml. 8%  $\text{CHCl}_3\text{COOH}$  at 0°C. to stop reaction. Protein precipitate removed by centrifuging and supernatant neutralized. Colorimetric reading of released inorganic P made exactly 5 minutes after adding Fiske-SubbaRow reagents. Blank determinations showed less than 1% hydrolysis of labile P of ATP under these conditions. Other aliquots removed after 30, 60 and 120 minutes at 30°C.

<sup>b</sup>  *$\beta$ -glycerophosphate, Fructose-1,6-diphosphate and Phytic Acid.* Since these compounds are stable, the release of inorganic P was followed by removing aliquots from the enzymatic reaction mixture at 0, 30, and 60 minutes. The aliquot was added to 1.00 ml. of 8%  $\text{CHCl}_3\text{COOH}$  to stop the reaction. After centrifuging off the precipitated protein, inorganic P was determined in the supernatant by the Fiske-SubbaRow method.

such dilute protein solutions, it is not possible to tell whether there is a small absorption at 260  $\text{m}\mu$  indicative of the presence of nucleic acid.

*Fraction II.* When completely free from Fraction I and sufficiently concentrated (1-2%), Fraction II has a slightly bluish color and this

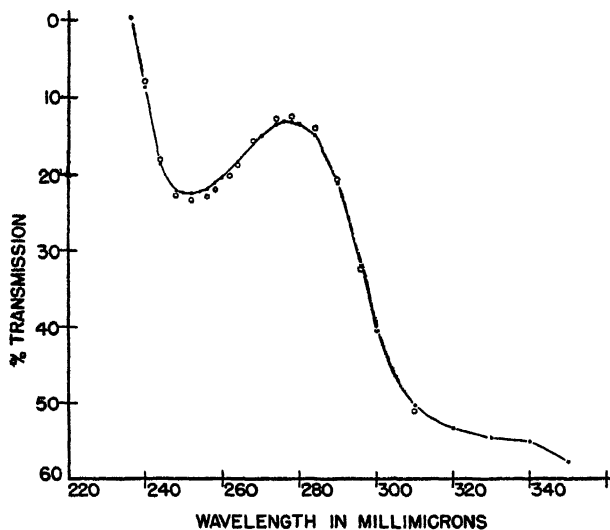


FIG. 7. The ultraviolet absorption spectrum of Fraction I. Samples containing 0.1 mg. protein/ml. of phosphate buffer were compared in a Beckman spectrophotometer. Solid dots, Fraction IA; open circles, Fraction IC.

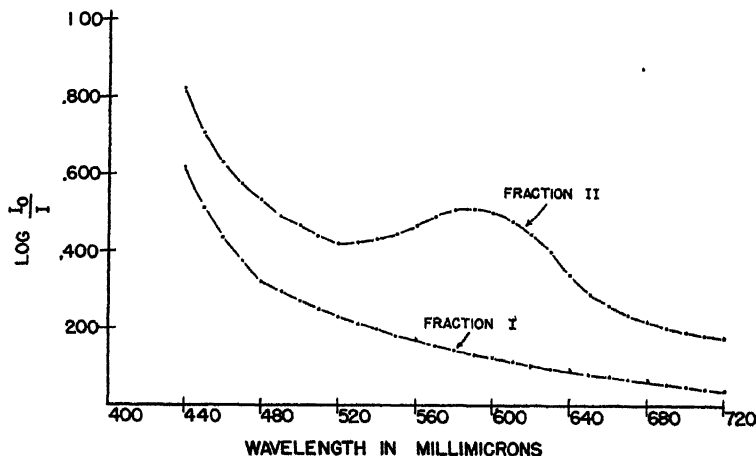


FIG. 8. The absorption spectrum of Fraction I and Fraction II in the visible region. Concentration of Fraction I, 1.0 mg. protein/ml. phosphate buffer; Fraction II, 2.0 mg./ml. phosphate buffer. Beckman spectrophotometer.



property has been found to be a useful guide in obtaining Fraction II. Even small amounts of Fraction I, however, can almost completely obscure the characteristic color of Fraction II. It should be pointed out that the protein responsible for the blue color tends to precipitate slowly on electrodialysis. Hence, close attention is required at this step in freeing Fraction II of Fraction I. When examined in a Beckman spectrophotometer, Fraction II exhibits a broad absorption maximum in the region of  $580\text{ m}\mu$ , as illustrated in Fig. 8.

No attempt has yet been made to separate the protein mixture into separate components. Using Thunberg techniques described previously (3), it is possible to demonstrate the presence of isocitric, malic, glutamic and alcohol dehydrogenases in Fraction II. This mixture of proteins (Fig. 2) also contains an active polyphenol oxidase, catalase and peroxidase. The results of enzyme experiments with Fraction II will be the subject of a separate communication.

### DISCUSSION

This investigation of spinach leaf protoplasm has shown that the cytoplasmic proteins can be separated into two distinctly different fractions by precipitation with ammonium sulfate. Of the total cytoplasmic proteins, approximately 75% appears to consist of a single, electrophoretically homogeneous protein (Fraction I). The other 25% consists of a mixture of proteins (Fraction II) as demonstrated by analysis in a Tiselius moving-boundary apparatus. Examination of these two fractions from the standpoint of enzymatic activities and content of bound auxin has led to the conclusion that essentially all of the bound auxin in spinach leaves is present in Fraction I, a protein which was also found to exhibit the enzymatic properties of a phosphatase. The auxin bound to Fraction I is similar in chemical properties to IAA.

Fraction I as prepared does not have the following enzymatic activities: catalase, peroxidase, polyphenol oxidase; malic, isocitric, glutamic, and alcohol dehydrogenases; aconitase, and tryptophan-converting enzyme (24). These enzymes have, however, been found in spinach cytoplasm and some of them have been demonstrated to be contained in Fraction II, the protein fraction which does not contain bound auxin.

Two kinds of experimental evidence still suggest that Fraction I may not be a homogeneous protein: (a) The enzyme attacks a variety

of phosphorylated substrates, and (b) it has not as yet been possible to show that the protein contains as much as one mol. of auxin per mol. of protein. The apparent yield of auxin obtained from Fraction I under the conditions used in most of these experiments is equivalent to  $2 \times 10^{-5}$  mg. of IAA/mg. of protein, a value which would require a minimum molecular weight of  $9 \times 10^6$ . Osmotic pressure measurements have shown the protein to have a molecular weight of approximately  $2 \times 10^5$ . Hence, a large discrepancy appears to exist between the amount of auxin released and the amount ( $8.8 \times 10^{-1}$  mg. IAA/mg. of protein) required if one mol of auxin is associated with one mol of protein. In experiments designed to determine the optimum conditions for auxin release, larger yields of auxin have, however, been obtained

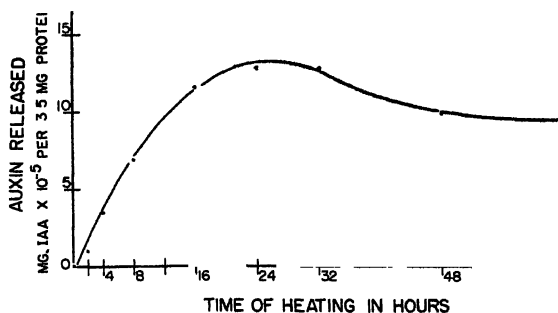


FIG. 9. The relationship between the amount of auxin liberated from Fraction I by alkali and the time of heating. 3.5 mg. of protein heated in a boiling water bath with 5.0 ml. of 0.1 *N* NaOH.

and as much as  $9.0 \times 10^{-5}$  mg. IAA/mg. of protein have been recovered. In addition, the release of auxin from Fraction I is evidently a complicated process as shown by the data in Fig. 9, results which are considered to be of a preliminary nature since experiments on the optimum conditions for the release of auxin from Fraction I will be the subject of a future communication. The data serve, however, to show that the release is not a simple, first-order reaction, but of a more complex nature, since the auxin released rises to a maximum at 24 hours and again disappears as the time of heating is extended. That IAA is indeed destroyed under the conditions used for the release of auxin from Fraction I is clearly shown by the data in Table XIII. Various

TABLE XIII

*The Effect of Adding Tryptophan and Indoleacetic Acid  
During Hydrolysis of Fraction I by Alkali*

All samples heated 8 hours in a boiling water bath in the  
presence of 5.0 ml. of 0.1 N NaOH

Wt of Fraction I in mg.	Addendum	Mg. IAA $\times 10^{-5}$ recovered	Per cent recovery of added IAA
9.2	—	12.9	—
9.2	1.0 mg. tryptophan	12.9	—
3.2	2 $\gamma$ IAA	22*	11
3.2	1 $\gamma$ IAA	39*	39
3.2	0.5 $\gamma$ IAA	18 <sup>+</sup>	45
3.2	0.25 $\gamma$ IAA	9 <sup>+</sup>	45

\*  $5 \times 10^{-5}$  mg. IAA have been deducted as auxin contributed by hydrolysis of protein.

amounts of auxin were added to Fraction I and the samples heated for 8 hours in a boiling water bath in the presence of 5.0 ml. of 0.1 N NaOH. In all cases, extensive inactivation of the added auxin occurred, the degree of inactivation depending upon the amount of IAA added. Thus, when 2  $\gamma$  of IAA were heated together with 3.2 mg. of Fraction I, 89% of the added auxin was inactivated. Similarly, with 0.5 or 0.25  $\gamma$  of IAA added to the protein, 55% inactivation occurred. It is, therefore, evident that no great reliance can yet be placed on the quantitative aspects of the present determinations of the auxin content of Fraction I.

Gordon and Wildman (9) have raised the question as to whether tryptophan contained in proteins may yield auxin and hence appear as a source of bound auxin when protein is heated in the presence of alkali, since they found that auxin was formed from tryptophan in the presence of hot alkali. Maximum conversion of tryptophan to auxin occurred when the amino acid was boiled for 7 hours in the presence of 0.0005 N NaOH, and auxin equivalent to  $5.7 \times 10^{-5}$  mg. IAA was formed from 1.25 mg. of tryptophan under these conditions, representing  $4.5 \times 10^{-3}\%$  conversion. Stronger or weaker base greatly reduced the amount of auxin converted from tryptophan.

When 10 mg. of Fraction I are heated in the presence of 0.1 N NaOH for 8 hours in a boiling water bath, auxin equivalent to  $18.8 \times 10^{-5}$  mg. IAA is released, or a yield of  $1.9 \times 10^{-3}\%$  based on weight of protein. Assuming as a first approximation that the tryptophan content of Fraction I is 2%, the figure found by Lugg (26) for the tryptophan content of spinach cytoplasmic proteins, then the amount of auxin to be expected by conversion of the tryptophan contained in 10 mg. of Fraction I would be only  $0.9 \times 10^{-5}$  mg.,  $0.9 \times 10^{-4}\%$  on the basis of protein weight, or less than one-

twentieth of that actually observed even assuming the maximum tryptophan conversion yet obtained.

Expressed in another way, at least 50% of the total protein sample would have had to consist of tryptophan to account for the amount of auxin released by the alkali treatment. Other evidence against the view that auxin released from Fraction I is a conversion product formed from tryptophan is found in Table XIII where it is shown that addition of tryptophan to Fraction I during hydrolysis does not increase the amount of auxin released from the protein. It would appear unlikely, therefore, that tryptophan conversion can account for more than a minor portion of the auxin obtained from Fraction I.

It should be stressed that final identification of auxin as an active and significant component of Fraction I can be obtained only if experiments designed to remove or lower the auxin content of Fraction I yield preparations of lowered enzymatic activity which can be restored by addition of auxin. So far, it has not been possible to remove auxin from Fraction I except by rather drastic means which inevitably result in irreversible denaturation of the protein as evidenced by loss of enzymatic properties.

Since Fraction I constitutes such a large portion of the cytoplasmic proteins, the possibility was considered that Fraction I might be a mixture of several proteins with identical or nearly identical mobilities and chemical properties. Several attempts were made to either dissociate an auxin-containing component or to remove the phosphatase activity of Fraction I by repeated, fractional precipitation with  $(\text{NH}_4)_2\text{SO}_4$ . All experiments of this kind failed to reveal any evidence for such a mixture. There was no indication that auxin enrichment or the converse occurred upon reprecipitation, nor was there any indication that the phosphatase activity was lost even though more than 75% of the initial starting protein was discarded in the process of obtaining four precipitations. Forty *per cent* of the phosphatase activity of whole cytoplasm was lost with the first precipitation, a loss which might conceivably be ascribed to the simultaneous loss of a second phosphatase associated with Fraction II, but thereafter, the change in activity with each successive precipitation was small and more indicative of slight denaturation of the protein rather than the specific loss of an enzymatic component. Furthermore, the homogeneous character of the Tiselius scanning patterns was not changed by repeated precipitation. Neither

the mobility of the protein nor the shape of the scanning pattern was altered in any significant way by the precipitation process.

The fact that the protein is capable of hydrolyzing such a variety of phosphorylated substrates might also be taken to indicate inhomogeneity, but it should be pointed out that no attempt has yet been made to follow the fate of these several activities as the protein is repeatedly precipitated, and, in addition, little is known about the specificity of purified phosphatases (19).

The phosphatase of Fraction I resembles that described by the histological experiments of Yin (25) for *Lamium* and *Iris* as well as for tissues of other plants. The similarity extends to pH optimum, reaction toward fluoride and magnesium, and substrate specificity in so far as comparison can be made. It is interesting to note that this phosphatase was found to be particularly active in meristematic tissues undergoing rapid growth, a fact in accord with the general idea that phosphatase activity may be of significance in growth. The phosphatase of Fraction I differs in several respects from the adenylypyrophosphatase studied by Kalekar (14) in the potato tuber. Not only was the potato enzyme fully soluble in 0.6 saturated ammonium sulfate, but also it showed a distinct pH optimum at pH 6.5. The Kalekar enzyme is also activated by Ca while the enzyme of Fraction I is not.

The difficulties involved in establishing the homogeneity of Fraction I are similar in many respects to the problems experienced with myosin from muscle. Myosin constitutes up to 70% of the total proteins of muscle, according to Bailey (1). Fraction I constitutes 75% or more of the total proteins of spinach cytoplasm. Both proteins display electrophoretic homogeneity to a greater degree than would be expected for a mixture of proteins. Both proteins have the ability to hydrolyze ATP,<sup>6</sup> and in neither case is it possible to demonstrate separation of this activity from the bulk of protein in undenatured preparations, although it is characteristic of both proteins to undergo a slight loss in phosphatase activity during repeated precipitation with ammonium sulfate.

<sup>6</sup> The turnover numbers of the two proteins in regard to ATP are remarkably similar. 1.8 mg. of thrice precipitated rabbit myosin can hydrolyze 40% of the labile phosphate from 10 mg. of ATP in 30 minutes at 37°C. With the same amount of substrate and enzyme, Fraction I of spinach cytoplasmic protein can hydrolyze 60% of the labile phosphate of ATP in the same time. The pH optima of these two proteins are quite different, however. Myosin works most rapidly at pH 9.0, Fraction I at pH 5.25.

Bailey's description of the difficulties in establishing the identity of ATP-ase and myosin might also apply to Fraction I.

He states (1), ". . . In general, an individual enzyme, whether of plant or animal origin, contributes little to the total weight of the whole organism. . . . Myosin constitutes some 57-70% of the total protein of muscular tissue. . . . Such a protein, acting as an enzyme and occurring in such vast amounts in the animal body, introduces a somewhat revolutionary change in our conception of the nature of organic catalysis. . . . It is possible to establish a reasonable case for an identity of this kind only by the massive accumulation of evidence which is mostly negative in character."

The same arguments apply to the present case. It would seem surprising that one protein with one type of enzyme activity should constitute such a large bulk of the cytoplasmic proteins. Actual identity of the protein with the phosphatase can be claimed only after much further investigation.

No final conclusion as to the role of auxin in Fraction I can be drawn at the present time, particularly in view of the fact that the exact amount of auxin bound in the protein cannot yet be determined with certainty. The data which have been presented would seem to suggest, however, that a reasonable working hypothesis may be that the auxin of the protein is related in some way to the specific enzymatic activity of the material. On this basis, probably the bound auxin of Fraction I should not be regarded as a precursor or storage form of auxin, but rather as the biochemically active form of the growth substance. Formation of auxin has been shown to take place in the spinach leaf through the intervention of an enzymatic mechanism by which tryptophan is transformed into an active growth substance, and tryptophan can accordingly be regarded as the auxin precursor in this plant (24).

#### ACKNOWLEDGMENTS

We wish to thank Dr. Stanley Swingle of the Division of Chemistry of this Institute for his courtesy in allowing us to freely use the Tiselius electrophoresis apparatus and the high speed centrifuge. The work would not have been possible without his consideration. We also wish to thank Dr. A. Goetz of the Physics Department for permitting us to use the Charlotte colloid mill throughout this work.

Dr. Ralph G. H. Siu of the Quartermaster Depot in Philadelphia participated in the early experiments on the fractionation of spinach protoplasm and we greatly appreciate his help. Mr. Victor Schocken has assisted on some of these experiments and we are also indebted to him for the molecular weight determination of Fraction I. Miss Martha Kent offered invaluable assistance on many of the *Avena* tests.

## SUMMARY

1. Methods are described for the separation of the protoplasmic constituents of spinach leaves and for the separation of the cytoplasmic proteins.

2. The spinach leaf cytoplasmic proteins can be separated into two principal fractions. The first fraction constitutes 70–80% of the total cytoplasmic proteins and appears to be electrophoretically homogeneous (Fraction I). The second fraction is electrophoretically inhomogeneous (Fraction II) and appears to include a variety of individual proteins.

3. Fraction I yields auxin on treatment with alkali or proteolytic enzymes. This bound auxin resembles indoleacetic acid since it is stable to hot alkali and sensitive to hot acid. The other proteins of the spinach leaf appear to contain small or negligible amounts of bound auxin.

4. Spinach cytoplasm has been shown to contain a large number of different types of enzymatic activities which are with one exception associated with the proteins of Fraction II. Enzymatic activities identified include four dehydrogenases, peroxidase, catalase, polyphenol oxidase and others. Fraction I, on the other hand, possesses only phosphatase activity and is able to hydrolyze bound phosphate from  $\beta$ -glycerophosphate, fructose-1,6-diphosphate, adenosine triphosphate, creatine phosphate, phytic acid and fructose monophosphate.

5. Both bound auxin and phosphatase activity appear to be integral properties of Fraction I and are not dissociable from this protein by repeated fractional precipitation of the protein with ammonium sulfate. The electrophoretic properties of the protein are also unaffected by repeated precipitation.

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## Subtilin Production in Surface Cultures

J. C. Lewis, R. E. Feeney, J. A. Garibaldi, H. D. Michener,  
D. J. Hirschmann, D. H. Trauffer,\* A. F. Langlykke,\*  
H. D. Lightbody, J. J. Stubbs and H. Humfeld

*From the Western Regional Research Laboratory,<sup>1</sup> Albany, Calif., and the  
Northern Regional Research Laboratory,<sup>1</sup> Peoria, Ill.*

Received April 15, 1947

### INTRODUCTION

Subtilin (1, 2, 3, 4), an antibiotic material produced by a particular strain of *Bacillus subtilis*, has been obtained in good yields in this Laboratory from a variety of culture media during the past two years. This paper describes the production of subtilin in shallow-layer surface cultures, as determined by the antibiotic activity of crude extracts, with emphasis on the use of media prepared from press juice concentrates from waste asparagus butts. The concentration and purification of subtilin, the assay method, and the microbial production in submerged cultures are described elsewhere (5, 6, 7).

### MATERIALS AND METHODS

The culture of *B. subtilis*<sup>2</sup> used was originally obtained from N. R. Smith, Bureau of Plant Industry, United States Department of Agriculture, as No. 231 and is now in the collection of the Northern Regional Research Laboratory, Peoria, Illinois, under the number B-543. Inoculum was grown on 8% asparagus or other medium through one or more transfers from a freshly grown slant culture. The strongly coherent pellicle obtained after 24-30 hrs. of incubation was dispersed with a sterile Waring Blendor or by shaking with sterile glass beads. One or 2% of this inoculum was generally used.

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\* Northern Regional Research Laboratory.

<sup>1</sup> Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture.

<sup>2</sup> The stock for several years has contained two phases which, on plating, yield colonies distinguishable by firm adherence and non-adherence to agar. Subcultures of adherent colonies produced rugose growth while non-adherent colonies produced mucoid growth on nutrient, tryptone-glucose, and asparagus agar. On similar broths

Asparagus butt juice media were prepared as described earlier (9). Most work was done with a concentrate (1944 season) prepared by the "digestion" process (9, 10). It contained 64% sugar and 3.5% non-ammonia nitrogen (dry basis). It was customarily diluted to 8 or 10% solids. Beet molasses medium was prepared by adding one part of beet molasses to 3 parts of tap water and supplementing with 0.8% diammonium phosphate and 50 p.p.m. of manganese as sulfate. The medium contained about 20% solids, 15% total sugar, and 0.50% non-ammonia nitrogen. Grain and molasses worts were obtained from a local bakers' yeast plant.<sup>3</sup> The grain wort contained about 10% solids, 6% total sugar, and 0.25% non-ammonia nitrogen. The molasses wort contained about 24% solids, 15% total sugar, and 0.50% non-ammonia nitrogen. Corn steep medium was prepared by diluting corn steep liquor concentrate with tap water and adding glucose and inorganic salts. An asparagine-sucrose synthetic medium (2) and modifications were also tested. Media were generally steamed for 20 or 30 minutes at approximately pH 5 and were neutralized, after cooling, with sterile sodium hydroxide.

Fernbach flasks with 500 ml. of medium (18 mm. depth) were used for culturing, except where otherwise indicated. Preliminary experiments were made with 50 ml. quantities of medium in 250 ml. Erlenmeyer flasks (11 mm. depth). Production runs were made with Fernbach flasks and with open aluminum pans of 4 and 6 square feet surface area. The cultures were usually incubated in a room held at 35°C. for 13 days.

A modification of McMahan's (11) turbidimetric method for penicillin was used for the assay of subtilin. *Micrococcus conglomeratus* (MY) was used routinely and most of the data presented in this paper were obtained with this organism. *Staphylococcus aureus* (H) and *Streptococcus faecalis* (ATCC 7080) were used occasionally. The method is described in detail elsewhere (6).

Samples were prepared for assay by adding 50 ml. of blended whole culture or culture liquor to 150 (occasionally 100) ml. of 95% ethanol. Pellicles were prepared for assay by straining off the culture liquor, after which the pellicles were blended with ethanol and water calculated to give about 70% ethanol in a volume corresponding

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the adherent isolates produced firm pellicles, the non-adherent isolates produced poor or no pellicles within three days. Significant differences were not observed on microscopic examination. On continued frequent transfer of the original adherent stock on Medium II of Schmidt and Moyer (8), the proportion of non-adherent phase cells increased and gave cultures which could be washed readily from agar. For convenience, one such non-adherent stock, cultured on agar slants of Medium II and stored in the refrigerator between transfers, was used routinely instead of the original adherent stock. It is probable that, on transfer of this inoculum to liquid medium, pellicle growth resulted largely from the adherent phase cells present in this stock.

<sup>3</sup> The grain wort was prepared by germinating barley, wet grinding it with wheat, inoculating with *Lactobacillus delbrückii* and digesting, extracting the mash, and recovering the wort by filtration. Molasses wort was prepared by diluting 4 parts of beet molasses and 1 part of cane molasses, heating and adding diammonium and trisodium phosphates and filter-aid, filtering off the precipitate, and diluting to the desired concentration.

to the original culture volume. The samples were shaken for at least one hour at room temperature. They were stored in the refrigerator until assays were made within the next few days. An estimate of the variability in culturing and assaying may be had from the data for duplicate cultures shown in Fig. 1 for the beet molasses medium.

Yields (antibiotic activities by bioassay) are calculated in terms of an arbitrarily chosen partially purified lot of subtilin (lot L1263). This standard subtilin represents an approximately 100-fold concentration of the activity in a surface asparagus juice culture pellicle (dry basis). The isolation and character of this material are described elsewhere (5).

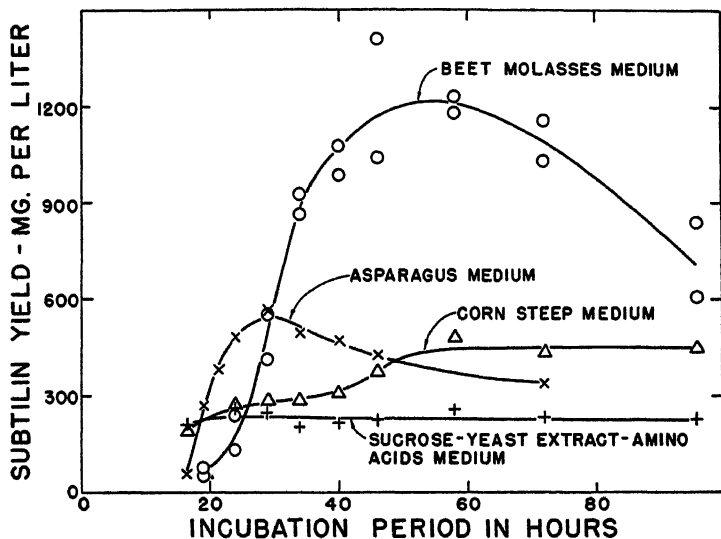


Fig. 1. Relation of subtilin yield to period of incubation. The media were composed as follows: Beet molasses 20% (dry basis)— $(\text{NH}_4)_2\text{HPO}_4$  0.8%—Mn 50 p.p.m.; Asparagus butt juice concentrate 10% (dry basis, 1944 season, digestion process); Corn steep liquor 4% (dry basis)—Glucose 4%—Mn 25 p.p.m.; Sucrose 10%—Yeast extract 0.4%—Asparagine 0.3%—Glutamic acid 0.3%— $(\text{NH}_4)_2\text{HPO}_4$  0.8%—K, Ca, Mg, and Mn 25, Fe and Mo 1, Cu 0.2, and Zn 0.25 p.p.m. *M. conglomeratus* was used as the assay organism.

## EXPERIMENTAL

*Yields on Various Media.* During the past two years subtilin yields have been determined for a variety of media. Close comparisons of yields are not warranted, however, since optimal cultural conditions and media composition were not established for individual media.

Asparagus concentrate (1944 season) prepared by a process permitting self-digestion of insoluble forms of nitrogen was markedly superior to one prepared from blanched asparagus butts, just as for tyrothricin production (9). The former gave yields of about 600 mg./l., the latter gave only 100–200 mg./l. Optimal yields were obtained at 10 or 13, but not at 7% solids in the former case, at 7.5 and 10, but not at 5% solids in the latter case. Unreported data with the 1944 season concentrate showed that the addition of 0.8% diammonium phosphate exerted an inhibitory effect, which was partially reversed by the addition of a mixture of Mn, Fe, and Cu. The addition to 10% asparagus medium of 0.4% of Bacto yeast extract, 0.7% Bacto casamino acids plus tryptophan, or both, increased subtilin yields about 200 mg./l. The further addition of 4% of glucose or sucrose was somewhat detrimental.

Molasses wort gave subtilin yields of 500–800 mg./l. The addition of 0.8% diammonium phosphate with or without additional salts appeared beneficial (unpublished data). The effect of concentration is shown by an experiment carried out in Erlenmeyer flasks (Table I),

TABLE I  
*Effect of Concentration on Subtilin Yields on Molasses Wort<sup>1</sup>*

Composition of medium			Subtilin yields by <i>S. aureus</i> after incubation for (hrs.):	
Total solids	Total sugar	Non-ammonia nitrogen	24	48
<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>mg./l.</i>	<i>mg./l.</i>
5	3	0.10	280	190
10	6	0.20	570	630
14	9	0.30	650	660
19	12	0.40	390	1100
24	15	0.50	110	1200

<sup>1</sup> Before dilution (24% solids), 0.8%  $(\text{NH}_4)_2\text{HPO}_4$  was added.

which contained shallower layers of medium and, therefore, gave higher yields than Fernbach cultures. The more dilute worts gave more rapid production; the more concentrated worts gave much higher but delayed yields. It may be noted that this strain of *B. subtilis* can tolerate high concentrations of sugar, unlike the strain of *Bacillus brevis* used for tyrothricin production (9).

The highest yields were obtained from beet molasses medium. Very heavy pellicles were obtained. In some cases less than half the

original volume of medium could be drained from the pellicle. The addition of both diammonium phosphate and manganese was necessary for maximum yields. In an experiment to determine the magnitude of the manganese requirement, beet molasses with or without the addition of 50 p.p.m. of manganese gave 70 mg./l. of subtilin after 48 hours of incubation. On beet molasses plus 0.8% diammonium phosphate the corresponding yields were 15, 35, 440, 790, and 950 mg./l. for the addition of 0, 5, 20, 50, and 100 p.p.m., respectively, of manganese. The beet molasses contained 6 p.p.m. of manganese, determined spectrographically, which yielded 1.2 p.p.m. in the diluted medium. The magnitude of the requirement on the molasses medium is noteworthy, since the asparagus media gave relatively good yields with manganese contents of about 2 p.p.m. Unpublished data showed that manganese could not be replaced by iron or copper.

Unsupplemented grain wort likewise proved a good medium, yielding about 600 mg. of subtilin/l. after 30–44 hours of incubation. The addition of various combinations of ammonium salts, phosphate, magnesium, manganese, and iron was without marked effect. A good yield was obtained after incubation for 3 days on a corn steep liquor medium (Fig. 1). Some unreported evidence indicates that the addition of salts was unnecessary. Poor growth and yields (less than 20 mg. of subtilin/l. in pellicle extracts) were obtained with media based on Bacto peptone, tryptone, or casamino acids plus glucose and salts. A low level of activity (under 60 mg./l. in 2–8 days) was obtained on the asparagine-sucrose-salts synthetic medium described previously (2) but this medium was greatly improved by adding Bacto yeast extract and other supplements (see Figs. 1 and 2). Preliminary results indicate that such a medium can be used as an assay medium for the factor(s) in beet molasses which is responsible for the high subtilin yields obtained from beet molasses medium.

*Effect of Time of Incubation.* The relation of subtilin yield to incubation time is illustrated for 4 media in Fig. 1. Cultures on the beet molasses medium were the slowest to start growing, but the subtilin yields attained after 2 or 3 days of incubation were the highest. The marked difference, with respect to loss of subtilin on prolonged incubation, between beet molasses and asparagus media on the one hand, and corn steep and sucrose-yeast extract-amino acids on the other, is noteworthy (see also Fig. 2).

*Effect of Incubation Temperature.* Growth of this organism on good media was accompanied by a marked production of heat during the period of rapid pellicle development. Fernbach cultures in air incubators began to heat up soon after pellicle formation was under way, and

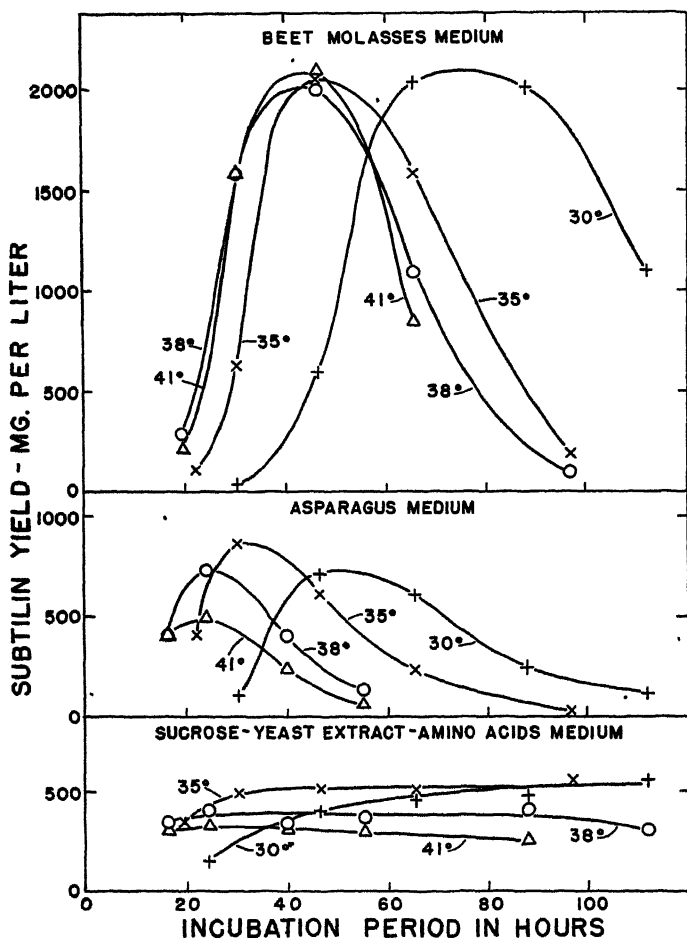


FIG. 2. Relation of subtilin yield to temperature of incubation. Constant temperatures were maintained by incubation of Erlenmeyer flask cultures in water baths. The media were the same as those cited in Fig. 1, except that 0.6% Bacto casamino acids plus 0.01% tryptophan were added to the sucrose-yeast extract-amino acids medium. *M. conglomeratus* was used as the assay organism.

culture temperatures 4–8°C. higher than incubator temperatures were quickly attained; that is, a flask in a 35°C. incubator might exhibit a temperature in the liquor of 39–43°C. Experiments were, therefore, conducted (a) with flask temperatures maintained constant by incubation in water baths and (b) with uncontrolled or partially controlled flask temperatures as could be obtained in an air incubator. The results of an experiment with Erlenmeyer flask cultures incubated in water baths are shown in Fig. 2. Growth and subtilin production began later at 30° than at 35°C. but in all three media substantially identical subtilin yields were attained with either temperature. Growth and subtilin production began sooner at temperatures somewhat higher than 35°C., but at sufficiently high temperatures the maximum subtilin yield suffered. This critical upper temperature limit varied for different media. It appeared to be between 35° and 38°C. for sucrose-yeast extract-amino acids medium, between 38° and 41°C. for asparagus medium, and above 41°C. for beet molasses medium. Thus, it appears that considerable latitude in choice of incubation temperature is available if the proper incubation time is chosen.

These results were extended by two experiments with Fernbach flasks. Molasses wort plus salts after 42 hours of incubation gave yields of 580, 570, 520, and 510 mg./l. for water bath temperatures of 34, 36, 38, and 40°C., respectively. In an air incubator at 38°C. a peak culture temperature of about 43°C. was attained without marked decrease in subtilin production. In an air incubator at 40°C., a peak culture temperature of about 45°C. was accompanied by a yield after 30 hours of incubation which dropped to 180 mg./l. on the molasses wort but was depressed less than 10% on grain wort plus 0.8%  $(\text{NH}_4)_2\text{HPO}_4$ . Thus, the critical upper temperature limit for molasses wort lay between 43° and 45°C., and above 45°C. for grain wort.

It proved possible, as anticipated, to start cultures at a higher temperature and then allow growth and subtilin formation to proceed at a reduced temperature. In such an experiment with molasses wort plus salts, Fernbach flask cultures were incubated at air temperatures of 35° and 38°C. until pellicle development was well under way, but subtilin formation was estimated to be less than one-third that found at harvest. At this time (27 hrs.) part of the flasks were cooled with water as much as 10°C. below control flask and 5°C. below incubator temperatures with no effect on subtilin yields at 44 hours. This experiment is of interest, in connection with tray culture production dis-



cussed below, in that it shows that precise temperature control during the later stage of incubation is unnecessary provided the temperature does not rise too high.

*Culture Vessels.* It was noted that yields expressed relative to the volume of culture medium were usually markedly higher for 250 ml. Erlenmeyer flasks than for Fernbach flasks (compare Figs. 1 and 2). This appears to be due principally to the use of an 11 mm. depth in the former and an 18 mm. depth in the latter case (Table II).

TABLE II  
*Influence of Depth of Medium, Culture Surface, and Size of Vessel  
on Subtilin Yields\**

Medium and inoculum	Depth of medium	Subtilin yields in:							
		250 ml. Erlenmeyer flasks (surface area 45 sq. cm.)		Fernbach flasks (surface area 330 sq. cm.)		Small enameled pans (surface area 800 sq. cm.)		Large aluminum pans (surface area 3,000 sq. cm.)	
	mm.	mg./l.	mg./1000 cm. <sup>2</sup>	mg./l.	mg./1000 cm. <sup>2</sup>	mg./l.	mg./1000 cm. <sup>2</sup>	mg./l.	mg./1000 cm. <sup>2</sup>
Molasses wort plus salts; inoculum from adherent stock	7			910	480				
	11	1010	1120	870	790				
	18			660	1000	490	920		
Beet molasses plus 0.8% (NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub> and 25 p.p.m. of Mn; regular inoculum	11	1400	1560	1620	1470	1970	2220		
	18	1160	2060	1080	1640	1100	2060	960	1790

\* All cultures were harvested after 44 hours of incubation.

The closer agreement for the beet molasses medium of yields expressed relative to surface area (Table II) suggests that, for this richer medium, subtilin production was limited more by surface available for pellicle development than by lack of nutrients.

*Sterilization.* It proved feasible to culture this rapidly and luxuriantly growing strain of *B. subtilis* on media which had only been steamed (at pH 5). Excellent growth and activity were even obtained on unsteamed asparagus, grain wort and molasses wort (15% sugar) media. Dilute molasses wort (6% sugar) could not be used without steaming since a gas from an unidentified contaminant bacillus raised the *B. subtilis* pellicle from the surface of the flask and markedly reduced growth and subtilin yield. Steaming was more convenient than autoclaving when large batches of medium were handled. Control experiments with autoclaved media gave similar results. Steamed media therefore were used routinely.

*Inoculum and Strain.* Variation in the level of inoculum from 0.5 to 5% were tested with a non-adherent stock culture on grain wort plus 0.8%  $(\text{NH}_4)_2\text{HPO}_4$  and with an adherent stock culture on dilute and concentrated molasses worts, plus salts. All subtilin yields fell within a 20% range for each medium, nor was there any marked effect on growth rate.

No difference in subtilin yield was found with 26-hour cultures on 8% asparagus medium when inocula from an adherent phase colony isolate and from a non-adherent stock were compared (158 and 164 mg./l. in the pellicle extracts). Similarly, inocula from non-adherent and adherent stocks gave comparable yields of subtilin on asparagus, grain wort plus 0.8%  $(\text{NH}_4)_2\text{HPO}_4$ , and dilute and concentrated molasses wort plus salts media. The pure non-adherent phase was not tested because of failure to form satisfactory pellicles.

Two other strains of *B. subtilis* available in this laboratory failed to produce significant amounts of a 70% ethanol extractable antibiotic effective against *S. aureus*. Evidence for the non-identity of subtilin and other antibiotics produced by other *Bacillus* species and *B. subtilis* strains is considered briefly elsewhere (5).

*Distribution of Subtilin Between Pellicle and Culture Filtrate.* Though early work (10) indicated subtilin was found principally in the pellicle, more extensive observations (Table III) have disclosed that the proportion of subtilin in the bacterial cells and in the medium varies widely from one medium to another though it is not markedly affected by various cultural conditions. The dependence of subtilin distribution on time of harvest was not investigated specifically, but it appeared

TABLE III  
*Distribution of Subtilin Between Pellicle and Culture Liquor*

Medium and other variables	Number of cultures <sup>1</sup>	Percentage of total subtilin found in pellicle
Concentrated molasses wort (24% solids) plus salts	24	88 (83-93)
Beet molasses plus 0.8% $(\text{NH}_4)_2\text{HPO}_4$ and 50 p.p.m. of Mn	11	85 (76-92)
Corn steep liquor-glucose-salts	13	80 (63-95)
Sucrose-asparagine-salts	4	70 (63-76)
Asparagus (1944 season, digestion process, 8-10% solids)	12	66 (45-86)
Sucrose-yeast extract-amino acids-salts	2	48 (44 and 52)
Diluted molasses wort (10% solids) plus salts	6	45 (37-57)
Grain wort plus salts		
pure adherent strain	7	40 (33-49)
mixed strain	15	26 (14-37)

<sup>1</sup> The cultures included various cultural and nutritional variations.

less important than in submerged fermentations (7). A much larger fraction of the total activity was found in the liquor in dilute than in concentrated molasses wort cultures. Inoculum from an adherent stock gave a slightly higher proportion of subtilin in the pellicle than inoculum from a non-adherent stock when grown on grain wort.

*Tray Production.* Substantial quantities of subtilin were produced in open, shallow, 2 feet square aluminum pans in a constant temperature room with provision for circulation and cooling of air, and provision for maintenance of the humidity near the saturation point. A high humidity was required to prevent evaporation and consequent concentration and lowering of the temperature of the medium. Cooling of the air was necessary after growth was well started since the temperature of the cultures would rise 5°–10°C. above the air temperature.

The media for such pan production runs were prepared in barrels, adjusted to pH 5.0, and held at 98°–100°C. for 30 minutes by injection of flowing steam. After cooling, pH adjustment, and inoculation, the medium was pumped into the clean but non-sterile pans. During the first part of the incubation period the room temperature was kept at 35°C., but during the latter part it was dropped to 30°C. Though contamination was encountered, runs with 550 liters of beet molasses medium disposed in 85 pans gave yields around 400–500 g. of subtilin.

## DISCUSSION

Various media from which crude subtilin activity can be obtained in good yield are given. It is likely that considerable enhancement of yields could be obtained by systematic study of supplementation and concentrations of various nutrients. It must be emphasized that the data given refer only to the antibiotic activity of crude culture extracts. The isolation of subtilin from surface cultures on asparagus medium is described in detail elsewhere (5).

Assays made with *M. conglomeratus*, *Staph. aureus* and *Strep. faecalis* have usually correlated well for various media and cultural conditions. Therefore it was possible to select one of the test organisms, *M. conglomeratus*, for routine use. Examples of parallel assays by the several test organisms are presented elsewhere (6).

## ACKNOWLEDGMENT

Thanks are due to Dr. E. C. Saudek of Consumers Compressed Yeast Company, Oakland, California, for grain and molasses worts and beet molasses, and Dr. S. K. Pilcher of Cutter Laboratories, Berkeley, California, for corn steep liquor. We are indebted to Mr. P. L. Blake for adaptation of an incubator room for pan production of subtilin, and to Dr. E. J. Eastmond for spectrographic analyses. We are also indebted to Mrs. E. M. Humphreys and Mrs. P. A. Thompson who performed the antibiotic assays.

## SUMMARY

Yields of subtilin obtained by growth of a particular strain of *Bacillus subtilis* (NRRL No. B-543) in shallow-layer cultures were determined by the antibiotic activity of crude aqueous ethanol extracts against *Micrococcus conglomeratus*, *Staphylococcus aureus*, and *Streptococcus faecalis*. The highest yields were obtained with a beet molasses medium, which required the addition of  $(\text{NH}_4)_2\text{HPO}_4$  (0.8%) and manganese (50 ppm.). Good yields were obtained on media prepared from asparagus butt waste press juice, molasses and grain worts, and corn steep liquor. Maximum yields were usually attained in cultures 1–2 cm. deep after 24–48 hours of incubation at an incubator temperature of about 35°C. Prolonged incubation of asparagus and beet molasses media resulted in a drop in antibiotic activity. An incubator temperature of 35°C., with peak temperatures in the cultures 4–8°C. higher, was approximately optimal. Lower temperatures resulted in slower growth and subtilin production, followed by a longer period before subtilin losses occurred. The quantity of inoculum was not critical. The distribution of subtilin between pellicle and culture filtrate varied widely for different media. Two other strains of *Bacillus subtilis* did not produce appreciable amounts of subtilin.

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# Subtilin Production in Submerged Culture

J. J. Stubbs, R. E. Feeney, J. C. Lewis, I. C. Feustel,  
H. D. Lightbody and J. A. Garibaldi

*From the Western Regional Research Laboratory,<sup>1</sup> Albany, Calif.*

Received April 15, 1947

## INTRODUCTION

Previous investigations (1, 2, 3) on the production of subtilin have been concerned with surface culture methods. Submerged culture methods, however, are usually preferable for large-scale production. This report deals with the production of subtilin by submerged culture techniques and factors which influence the yields of subtilin produced by a particular strain of *Bacillus subtilis* cultured on several media.

## MATERIALS AND METHODS

The strain of *B. subtilis* used in this and related investigations (1, 2, 3, 6) was designated in the stock culture collection of the Northern Regional Research Laboratory as B-543. The original stock produced a firmly adhering growth on agar. On continued transfer on "Medium II" of Schmidt and Moyer (4) it produced a growth which was easily removed from the surface of the agar. This "non-adherent" stock was used routinely largely because the growth could be washed easily from agar slants.

Inocula for routine studies were prepared by washing the growth from a Medium II agar slant, incubated at 35°C. for 24 hours, into 500 ml. of sterile medium dispensed in a Fernbach flask. The cultures developed a heavy pellicle during incubation for 24 hours at 35°C. The content of the flask was blended in a sterile Waring Blendor to obtain uniform aliquots of the cell suspension. From 50 to 100 ml. of this suspension were used to inoculate one liter of culture medium. In several experiments inocula produced under submerged culture conditions were used.

Detailed studies of the nutritional requirements of the organism for the production of subtilin were not made. Instead, media previously found to produce highest yields of subtilin in surface cultures (3) were employed. The asparagus medium was prepared by diluting asparagus butt juice concentrate (1) with tap water to the desired concentration of total solids (usually 8%). The compositions of other media tested are listed in the footnote of Fig. 3. Media were steamed in one liter aliquots at 100°C. at

<sup>1</sup> Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

atmospheric pressure for a period of 20 minutes at pH 5.5. The pH of the media was adjusted to 6.8–7.0 just prior to inoculation. The temperature of incubation was 35°C. and the air delivery was one volume of air/volume of medium/min. unless otherwise indicated.

A laboratory fermentor developed in this Laboratory for the study of yeast propagation was used in these studies (5). This equipment was found to be greatly superior to either gas washing bottles with sintered glass false bottoms or to shake flasks for either cell production or subtilin formation. The essential feature of the fermentor is high speed dispersion of the aerated culture by means of a propellor driven at 1,550 r.p.m.

The assembled fermentors were sterilized at 15 pounds pressure for twenty minutes, cooled and placed upright in a water bath. A one liter charge of inoculated medium was transferred aseptically to each fermentor. Air was admitted to the culture at a predetermined rate through the bottom of the fermentor and the stirrer started at once. Foaming commenced during the early stage of the fermentation. A few drops of "Vegafat Y" (National Oil Products Company) were added when necessary to prevent excessive foaming.

Samples of the culture medium were removed by means of a pipette attached to a rubber tube leading from the bottom of the fermentor. To insure representative sampling the pipette was filled and discharged back into the fermentor twice before a sample was removed.

Cell production was followed by determining the volume of bacterial cells present in the medium at various time intervals. Ten ml. of the cell suspension were centrifuged in Kohlmer tubes for a period of 20 minutes at 3,400 r.p.m. using an International Centrifuge, Size 1, Type SB, with a No. 225 head. In most instances the cells were firmly packed in the bottom of the tubes and were distinctly separated from the supernatant. Cell volumes so obtained were expressed as percentages of the total culture volumes. Another 10 ml. sample of the culture medium was diluted with 30 ml. of 95% ethanol, extracted for a period of one hour at room temperature on a shaking machine, and stored in a refrigerator pending bioassay. Aliquots of this extract were assayed against a standard sample of partially purified subtilin (Lot L1263) using *Micrococcus conglomeratus* as the test organism<sup>2</sup> by a procedure to be described elsewhere (6). Subtilin yields were expressed in mg./l. of culture medium as determined against this standard.

## RESULTS

*Quantity and Type of Inoculum.* Cell production and subtilin formation were initiated sooner with the non-adherent stock than with the original adherent stock, but the final yields of subtilin were not significantly different (900–1,000 mg./l.).

The quantity of inoculum required for subtilin production was not critical. Varying the quantities of inocula produced by submerged

<sup>2</sup> Comparative antibiotic activity of subtilin using several test organisms will be reported by Lewis *et al.* (6).

culturing from 1 to 4% did not influence the final yield of the antibiotic. As an example, a fermentor was inoculated with 100 ml. of a blended surface culture and incubated for 5.5 hours, at which time the cell volume was 13%. A portion of this culture was then withdrawn aseptically from the fermentor. Three other fermentors were then inoculated with 10, 20, and 40 ml., respectively, of this culture and incubated for 7 hours. Yields of subtilin in the respective fermentors were 1,050, 1,080, and 1,040 mg./l. In other experiments, employing varying quantities of blended surface cultures as inocula, similar results were obtained.

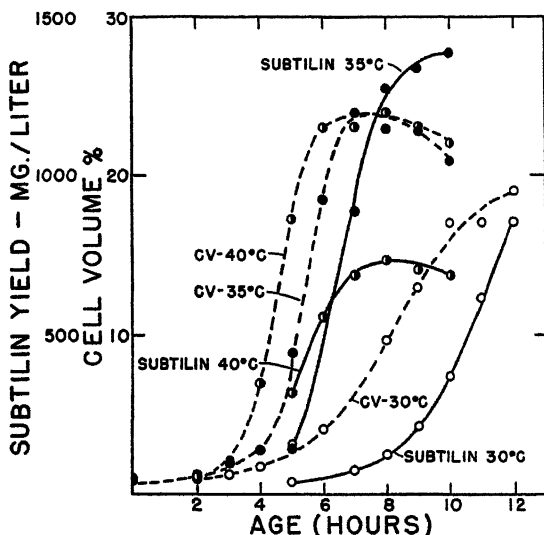


FIG. 1. Effect of temperature on growth and subtilin production.

*Effect of Different Incubation Temperatures.* Results of an experiment in which the fermentors were operated at different temperatures are shown in Fig. 1. The best results were obtained at 35°C. Although a high cell volume was rapidly obtained at 40°C, the subtilin content of the medium at the end of 10 hours was only half that produced at 35°C. (700 mg./l. as compared to 1,370 mg./l.). At 30°C. cell production and subtilin production were much slower than at 35° or 40°C.

Since the process is exothermic during the rapid growth phase, the temperature of the medium rises substantially unless adequate pro-



visions are made for the removal of heat. The importance of adequate temperature control in processes involving large-scale production is obvious.

*Effect of Air Flow on Subtilin Production.* One liter of air/min./l. of culture was near optimum. Subtilin yields of 1,030, 1,130, and 520 mg./liter were obtained when the air flow was maintained at 2, at 1, and at 0.2 l./min., respectively. At the highest air flow more difficulty was encountered in controlling foam and in obtaining representative samples of culture medium for assay. It is possible that the oxygen requirements during the initial stages of operation are lower than employed in these experiments, but a study of this factor was not made. Early in the investigation it was noted that a short (10–20 min.) interruption of the fermentation process by either shutting off the air supply or stopping the dispersing propellers resulted in poorer yields of subtilin. It was, therefore, mandatory that no such interruption occurred.

*Effect of pH.* In an experiment on asparagus juice medium of 8.0% solids, a medium with an initial pH of 7.0 gave a maximum yield of subtilin only 16% less than the yield obtained on a medium with an initial pH of 6.6. Other experiments, in which the initial pH was inadvertently varied within this range, resulted in differences in yields of less than 10%.

On asparagus juice medium the pH invariably decreased during the growth period, sometimes to as low as 5.7. Toward the peak of cell volume the pH rose again, frequently above 7.0.

*Concentration of Asparagus Medium.* Asparagus media with solids contents of 8, 10.9, and 13.6% were studied. The results are given in Fig. 2. Both subtilin and cell productions were slower as the solids were increased, but higher yields of antibiotic were obtained at the higher concentrations. However, the lower levels (8 and 10.9%) were usually used, since the lag phase was considerably less, thus decreasing the time necessary for the completion of an experiment.

*Other Media.* In Fig. 3 are given the results of subtilin production studies on 3 different media under comparable submerged culture conditions. Productions on these media were radically different. Subtilin production was the most rapid on the yeast extract-sucrose-amino acid medium but ceased after 7 hours of incubation with a maximum yield of 620 mg./l. Subtilin production was slightly slower on the asparagus juice medium but continued until 10 hours, at which time the yield was 1,530 mg./l. In contrast to the above, production on the beet molasses medium was much slower. After 14 hours of incubation the yield of subtilin was 1,230 mg./l. and apparently was still

increasing. The yeast extract-sucrose-amino acid medium used in these experiments was not optimum and undoubtedly contained superfluous materials. The results obtained with this medium are presented merely for illustrative purposes.

*Rate of Production.* Very heavy growth and high antibiotic yields were rapidly obtained in these studies. During periods of active growth the cell volumes more than doubled each hour. The data of the 35°C.

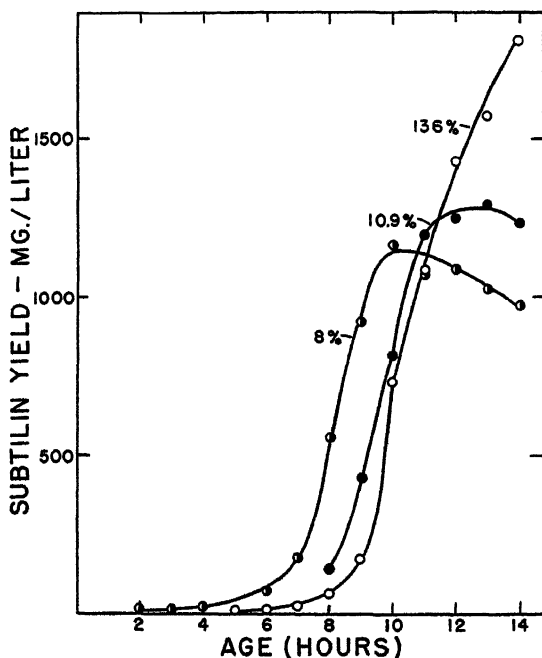


Fig. 2. Effect of concentration of asparagus juice on subtilin production. Concentrations of media expressed as per cent solids.

fermentor in Fig. 1 illustrates this. The cell volume increased from 3.8% at 4 hours to 9.0% at 5 hours, and to 18.5% at 6 hours (at times, cell volumes as high as 35% were obtained on asparagus juice media). The subtilin production increased from 140 mg./l. at 5 hours to 880 mg./l. at 7 hours, and 1,260 mg./l. at 8 hours. Thus, the subtilin content increased 9-fold with a synthesis of 1,120 mg./l. of antibiotic in the 3-hour period.

Subtilin formation lagged behind cell production from one to two hours (Fig. 1). In the majority of the experiments on asparagus juice media a 40-60% increase in the yield of subtilin was obtained by operating the fermentors for two additional hours after a maximum

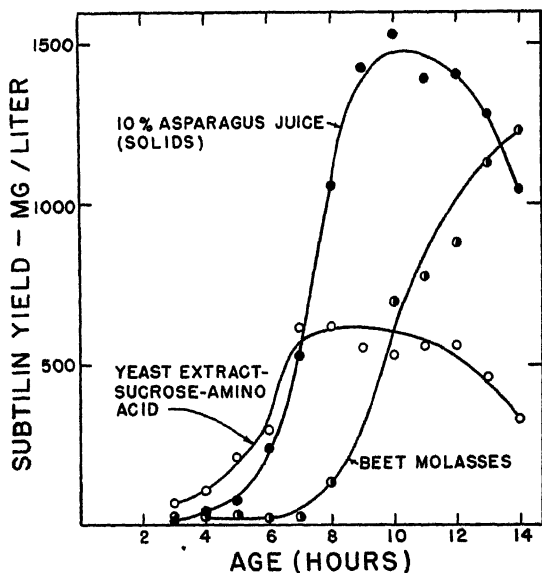


FIG. 3. Subtilin production on different media. *Asparagus Juice Medium*: Asparagus concentrate (68% solids), 147 g.; tap water to make one liter of solution. *Beet Molasses Medium*: Beet molasses (82% solids), 250 g.;  $(\text{NH}_4)_2\text{HPO}_4$ , 8.0 g.;  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 0.15 g.; tap water to make one liter of solution. *Yeast Extract-Sucrose-Amino Acid Medium*: Sucrose, 100 g. Asparagine, 3 g.; Glutamic acid, 3 g.; Casamino acid, 6 g.; Yeast extract, 4 g.;  $(\text{NH}_4)_2\text{HPO}_4$ , 8 g.;  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 0.15 g.; Tryptophan, 0.1 g.; Conc.  $\text{H}_2\text{SO}_4$ , 3 ml.; Salt mixture, 10 ml.; Distilled water to give one liter of solution. The salt mixture contained the following salts/l. of solution:  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ —0.97 g.;  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ —0.72 g.;  $\text{MoO}_3$ —0.15 g.;  $\text{CuSO}_4$ —0.5 g.;  $\text{ZnCl}_2$ —0.21 g.;  $\text{CaCl}_2$ —13.9 g.;  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ —41.8 g.;  $\text{KCl}$ —9.6 g.

cell volume was obtained. Further incubation resulted in a loss of subtilin. It was, therefore, necessary for maximum yields to harvest cultures approximately 2 hours after maximum cell volumes were obtained.

*Distribution of Subtilin Between Cells and Medium.* In Table I are listed the subtilin contents of the cells and cell-free medium at several

TABLE I  
*Distribution of Subtilin between Cells and Medium<sup>1</sup>*

Age of culture	Cell volume	Subtilin in one liter of culture			Subtilin concentration		
		Whole culture	Cell fraction	Cell-free fraction	Cell fraction	Cell-free fraction	Ratio <sup>2</sup>
<i>hrs.</i>	<i>per cent</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg./l.</i>	<i>mg./l.</i>	
7	21	410	280	130	1330	165	8.1
10	16	690	340	350	2130	417	5.1
12	18	740	315	425	1750	518	3.4

<sup>1</sup> The medium of this experiment consisted of 8% (solids) asparagus juice diluted with tap water.

<sup>2</sup> The ratio is the quotient of the concentration of subtilin in the cells divided by the concentration in the cell-free fraction

stages during a submerged run. Though there was more total subtilin in the cell-free medium than in the cells at the end of the experiment, the actual concentration of subtilin in the cells was at all times higher than in the medium. After 7 hours' incubation there was 8.1 times as much subtilin/ml. of cells as/ml. of the cell-free medium, and 3.4 times as much after 12 hours. At the twelfth hour of fermentation slightly more than one-half of the total subtilin was in the cell-free medium.

## DISCUSSION

The rate of growth of bacteria has been an extensively investigated subject. The successive measurements of cell volumes as followed in this work do not accurately determine rates of cell multiplication, rates of increase in cellular substance, or exact amounts of cellular substance present in the culture at any one time. However, the cell volumes so obtained were probably comparable to one another and served as a convenient method of determining harvest times.

Though the actual weight of subtilin produced in these studies could not be determined, since the purity of the standard employed for assay was unknown (6), the amount was probably considerable and the rate of formation rapid. Studies of subtilin formation on synthetic media under submerged culture conditions should yield interesting information on factors influencing this rate and, in particular, the rapid conversion of possible precursors.

The short lag between cell production and the appearance of subtilin in the culture may be variously interpreted. The antibiotic may be a degradation product of some component of the bacterial cell or a slight modification of such a material. From the fact that more antibiotic is found associated with the cells than in the cell-free medium during the time of rapid cell growth, one might conclude that the antibiotic is produced either within the cell or at the surface of the cell. The fact that, on some media, the subtilin apparently begins to disappear at an appreciable rate after having passed through a maximum level indicates the possibility that, under certain submerged cultural conditions, there may be a simultaneous synthesis and inactivation of subtilin. The increasing quantities of the antibiotic which appear in the media with time are probably due to cell autolysis. The observed inactivation which occurs after the maximum cell volumes are attained are a possible consequence of partial loss of cellular organization.

The deleterious effects of interrupting the fermentations by shutting off the air supply or stopping the dispersers is in accordance with the high oxygen requirements of bacilli as reported by Rahn (7).

Relative yields of subtilin obtained under submerged culture conditions may differ markedly from those obtained by surface cultures (3). On beet molasses media yields of subtilin of 1,100–1,300 mg./l. were obtained in both submerged and surface cultures (3). In contrast to this, submerged cultures on asparagus juice media produced more than twice the subtilin obtained by surface cultures (1,000–1,400 mg./l. as compared to 500–600 mg./l.). The antibacterial properties and identity of the materials prepared by the two methods of culturing have not yet been investigated.

#### ACKNOWLEDGMENT

The authors are grateful to John R. Hoffman and Ernst Aeschlimann for technical assistance in the construction and servicing of the fermentors, and to Elizabeth M. Humphreys and Precious A. Thompson for the antibiotic assays.

#### SUMMARY

Excellent yields of subtilin were produced by submerged fermentation on asparagus butt juice. Fermentors of one liter capacity equipped with high speed stirrers for dispersion of air were found to be much

superior to gas washing bottles with sintered glass false bottoms or to shake flasks. Asparagus juice concentrate, when diluted to 8% solids, steamed for 30 minutes, adjusted to pH 7.0, inoculated and incubated at 35°C. under aeration at a rate of one volume of air per volume of medium per minute, yielded 1,000–1,200 mg. of subtilin/l. of culture liquor after a 10-hour incubation period. Subtilin was also produced in similar yields but at a slower rate from media prepared from beet molasses.

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# The Microbiological Assay of Subtilin

J. C. Lewis, E. M. Humphreys, P. A. Thompson,  
K. P. Dimick, R. G. Benedict,<sup>1</sup> A. F. Langlykke<sup>1</sup>  
and H. D. Lightbody

From the Western Regional Research Laboratory,<sup>1a</sup> Albany, Calif., and the  
Northern Regional Research Laboratory,<sup>1a</sup> Peoria, Ill.

Received April 15, 1947

## INTRODUCTION

Assays in connection with investigations in this Laboratory of production (1, 2) and purification (3) of subtilin were made by a turbidimetric bacteriostatic method involving 4- or 5-hour incubation of assay cultures. This paper describes the method used, which was based on McMahan's (4) assay for penicillin. Data are also presented which demonstrate heterogeneity of subtilin activity in crude extracts and partially purified preparations.

## METHODS

*Cultures and Inoculum.* Most assays were made with *Micrococcus conglomeratus* (MY) or *Staphylococcus aureus* (II). Occasional comparisons were made with *Streptococcus faecalis* (N.R.R.L. B-537, A.T.C.C. 7080). Stock and working cultures of all three bacteria were maintained on Schmidt and Moyer's (5) medium II<sup>2</sup> agar. Working cultures were transferred weekly, incubated at 37°C., and stored in the refrigerator. Inoculum was grown by transferring a loop of cells from a slant culture to 150 ml. of medium II and incubating overnight at 37°C. It was added to the assay medium in amounts required to give 4% for *M. conglomeratus* and *S. faecalis*, and 2.5% for *S. aureus* in the final assay cultures.

*Assay Media.* *S. aureus* and *S. faecalis* were grown on medium II. *M. conglomeratus* was grown on a medium containing 2% of trypsin-digested casein (N-Z-amine, Sheffield Farms) and 0.5% of yeast extract (Difco) plus the glucose and  $\text{KH}_2\text{PO}_4$  of

<sup>1</sup> Northern Regional Research Laboratory.

<sup>1a</sup> Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture.

<sup>2</sup> Peptone 0.5%, yeast extract 0.15%, beef extract 0.15%, glucose monohydrate 0.35%,  $\text{KH}_2\text{PO}_4$  0.4%, pH 6.8 with KOH.



medium II. Growth on this medium was considerably better than on medium II or a modified medium II (which was used in early assays) containing double concentrations of peptone, yeast extract, and beef extract. The media were prepared 50% more concentrated than desired in the assay cultures to allow for dilution by the aliquots of subtilin. The media were cooled to refrigerator temperature before use.

*Subtilin Standard.* Yields are calculated in terms of an arbitrarily chosen, partially purified lot of subtilin (Lot L1263). This standard represents an approximately 100-fold concentration of the activity in a 28-hr. surface asparagus juice culture pellicle (dry basis). It was isolated by a process similar to that described elsewhere (3) (see also footnote 1, Table V). The dry subtilin standard was stored in the refrigerator, since a slow loss of activity occurred on storage at room temperature (3). A solution containing 40  $\gamma$ /ml. of this standard in distilled water adjusted to pH 2.5 with HCl was stored in the refrigerator for routine use. It was renewed every 2-3 months, although it appeared to be fairly stable (3).

*Samples.* Aqueous culture samples were diluted with 3 volumes of 95% ethanol to give approximately 70% ethanol. After shaking at room temperature for at least one hour, the samples were ready for assay. Butanol extracts were diluted with 70% ethanol to facilitate solution in the aqueous diluent. A concentration of 0.5% of ethanol in the final assay culture had little effect on the response of the test organisms to subtilin. Such a concentration was attained only with very impotent samples. A 1:1 mixture of ethanol and butanol could be tolerated in the final assay culture only at a concentration of 0.1% or lower; consequently, only relatively potent butanol extracts could be assayed directly. Lyophilized subtilin preparations were dissolved in pH 2.5 HCl. Preparative fractions not fully soluble in pH 2.5 HCl were triturated with glacial acetic acid. On dilution, such samples frequently gave colloidal suspensions. They were diluted to approximately 40  $\gamma$  of activity/ml. with pH 2.5 HCl and gently rocked overnight in the cool room. Unneutralized acetic acid begins to interfere appreciably at a concentration of 0.05% in the final assay culture. All samples were stored in the refrigerator pending assay.

*Procedure.* The assays were conducted with 15-ml. cultures in 18  $\times$  150 mm. Pyrex culture tubes. It was not necessary to sterilize the culture tubes.

Aliquots of appropriate dilutions of standard subtilin and unknowns in pH 2.5 HCl were pipetted by means of a Brewer automatic pipetting machine (Baltimore Biological Laboratory). It was convenient to pipette 3, 2, and 1 aliquots (1.67 ml. each) for *S. faecalis* and *S. aureus*, and 4, 3, and 2 aliquots (1.25 ml. each) of standard for *M. conglomeratus* which gave a dosage-response curve with greater slope than those given by the first two organisms. The appropriate number of aliquots of pH 2.5 HCl were then added to bring each tube to 5-ml. Three replicates were run at each level of standard or unknown. Control tubes containing no subtilin, and others containing sufficient subtilin to completely inhibit growth were also provided.

Ten-ml. aliquots of cold inoculated medium were pipetted into all tubes in essentially the same order as that for the subtilin solutions. The delivery tube was held so that the sides of the tubes were rinsed down and the medium and test solutions were mixed without the necessity of shaking the tubes. The introduction of air bubbles into the medium was avoided, and the method of delivery was as uniform and as rapid as possible. The tubes were placed at once in a water bath at 37°C. and incubated with-

out disturbance for 4 hours with *S. aureus* and *S. faecalis*, and for 5 hours with *M. conglomeratus*.

It was convenient after incubation to cover the racks of tubes with cotton-lined stainless-steel covers and to sterilize them under steam pressure. Turbidity readings were made on the following day. A Klett-Summerson colorimeter equipped with a red filter and the vacuum emptying device described by McMahan was used. Subsequent calculations were simplified by zeroing the instrument against control cultures containing excess subtilin (culture medium blanks). The bacterial cells in cultures permitted to stand overnight were resuspended by inverting the culture tube several times, taking care to avoid formation of foam or stable bubbles. If it was desired to read the tubes immediately after incubation, the cultures were steamed at atmospheric pressure as recommended by McMahan (4), since the turbidity of cultures sterilized by steam pressure changes markedly during the reading period.

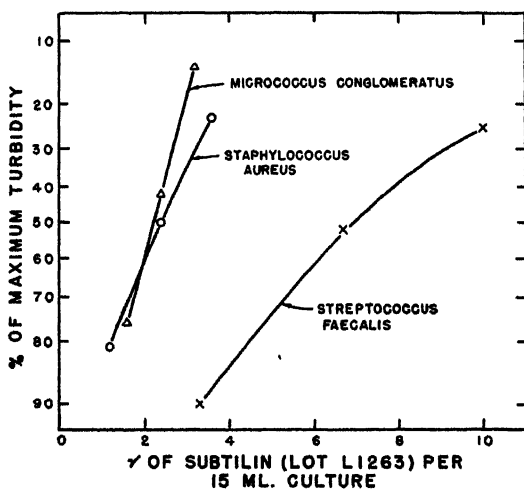


FIG. 1. Response of assay organisms to the standard preparation of partially purified subtilin.

**Calculations.** Since the sigmoidal dosage-response curves obtained were difficult to plot on rectangular coordinate paper from a limited number of points, the readings were converted to per cent of maximum turbidity (controls without subtilin) and the standard response was plotted on probability paper. This procedure gave approximately linear standard curves for *M. conglomeratus* and *S. aureus* (Fig. 1); an approximately linear curve was obtained for *S. faecalis* by use of logarithmic probability paper.

Considerable trouble was experienced in maintaining constancy of response to the standard throughout an assay experiment. Therefore, the standard solution was inserted in several positions throughout the assay experiment. Progressive shifts in

activity of the standard as great as 15% were frequently observed in experiments with about 20 samples. An arbitrary standard curve, approximating the mean curve, was drawn and the apparent activities of the standards as well as the unknowns were estimated. The assays of unknowns were then corrected proportionately to the apparent activities of the adjacent standards.

## RESULTS

Since early work indicated a high degree of variability in 18-hour incubation, serial dilution type assays for subtilin, the short incubation period turbidimetric method was adopted because of its promise of high precision. Replicate cultures prepared side by side have usually checked within 1 or 2 "Klett" units. This is good in view of the fact that turbidities of 60-100 units were customarily obtained in subtilin-free controls. Consequently, it was easy to recognize variations in apparent activities of identical samples in various positions in the assay setup, in relative activities of preparations assayed simultaneously on different days, in relative activities of preparations assayed with different test organisms, nonadditivity of response to individual preparations and mixtures of the same, and other effects discussed below.

Subtilin in dilute solution appeared to be relatively unstable in distilled water containing dissolved air as compared with freshly boiled distilled water or dilute acetic acid or HCl. Distilled water adjusted to pH 2.5 with HCl proved a convenient diluent for minimizing this instability, and was used routinely. The resulting effect on pH of the buffered basal media was negligible. Cysteine hydrochloride, ascorbic acid, and sodium bisulfite in 0.1% solutions were tested as stabilizing agents but proved unusable because of growth inhibition when tested on *M. conglomeratus* and *S. aureus*.

It was also found that the extent of inhibition by a given level of subtilin was markedly decreased if the mixture of inoculated medium and subtilin was permitted to stand at room temperature before placing in the bath for incubation. This phenomenon apparently influenced the relative activity of similar standards introduced at the beginning, middle, and end of the assay setup. The effect was minimized by the use of cold inoculated medium, and by reducing the time for setting up an assay to a minimum.

Turbulence in the bath had a detrimental effect on subtilin activity. Comparison of culture tubes shaken periodically during incubation

with undisturbed tubes showed that shaking resulted in about 30% less inhibition for a given level of subtilin. The necessity for avoiding any disturbance of the tubes during incubation is emphasized.

It was found that tubes sterilized in paper-lined metal containers in a gas oven gave erratic results, possibly a result of residues in the tubes from gas fumes or from carbonization of the lining paper. Unsterilized tubes gave satisfactory results and were used routinely. The necessity for constancy in the method of cleaning tubes is suggested by these results. Dichromate-sulfuric acid cleaning solution was used.

The consistency of data obtainable on certain soluble samples is illustrated by the results of a dialysis experiment given in Table I.

TABLE I  
*Activity and Material Balance for Dialysis of Subtilin*

Fraction	Potency of fraction	Wt. of fraction	Standard equivalent activity in fraction
	<i>per cent</i>	<i>mg</i>	<i>mg</i>
Starting material <sup>1</sup>	(100) <sup>2</sup>	5000	5000
0-18 hr. diffusate	109	1150	1250
18-24 hr. diffusate	111	1670	1850
49-90 hr. diffusate	94.5	1170	1110
Non-diffused residue	60.5	750	450
Weight unaccounted		260	260 <sup>3</sup>
sums		5000	4920
discrepancy			80 mg. or 2%

<sup>1</sup> Previously dialyzed subtilin (lot L1254) from 70% ethanol extract of asparagus juice culture pellicle, prepared by an adsorption-elution procedure (see footnote 3, Table V).

<sup>2</sup> Assays were made by *S. aureus* relative to the starting material which assays 25 50% as active as the regular standard (L1263), see discussion, p. 418.

<sup>3</sup> Assumed.

Equally good balances were obtained in several similar experiments. Less satisfactory results were obtained with overall isolation balances where certain fractions were not completely soluble and where all the fractions were not antibiotically similar; but, in general, balances have checked out within 10%.

*Extraction Conditions.* A concentration of about 70% ethanol was chosen for extraction of subtilin from *Bacillus subtilis* cultures, since it was known that subtilin is quite soluble in aqueous ethanol but

relatively insoluble in 95% or absolute ethanol (3, 6). On the other hand an extract containing 60% or less of ethanol generally does not flocculate readily to give a clear supernatant, even on centrifuging. Subsequently, it was found (Tables II and III) that the concentration

TABLE II  
*Extraction of Subtilin from Bacillus subtilis Cultures with Ethanol*

Assay organism	Concentration of ethanol	Surface culture (44 hrs. on 8% asparagus juice) blended thoroughly and adjusted to:			Submerged culture (13 hrs. on supplemented asparagus juice) adjusted to:		
		pH 7.1 (unadjusted)	pH 4.0 with acetic acid	pH 2.5 with HCl	pH 6.8 (unadjusted)	pH 2.5 with HCl	pH 2.5 with HCl plus 2% NaCl
<i>M. conglomeratus</i>	per cent		mg. of subtilin/l. of culture <sup>1</sup>				
	50	390 <sup>2</sup>	390 <sup>2</sup>	480	380 <sup>2</sup>	520	560
	60	410 <sup>2</sup>	150	520	420 <sup>1</sup>	560	560
	70	380	440	540	400	450	490
	80	310	300	460	380	440	440
<i>S. faecalis</i>	50	420	400	390	380	510	540
	60	450	500	510	440	520	490
	70	440	510	540	470	450	430
	80	370	400	530	420	370	420
<i>S. aureus</i>	50	500	470	580	480	570	560
	60	550	560	690	570	710	730
	70	510	580	740	630	650	710
	80	440	420	620	600	650	510

<sup>1</sup> Extracted for 2 hours with continuous shaking at 5 times the volume of the original culture aliquot.

<sup>2</sup> Slightly turbid extracts.

<sup>3</sup> Very turbid extracts even after centrifugation. Clarity is increased with increasing ethanol concentration and with acidification. Marked differences in ease of flocculation were found, in general, for different cultures.

of ethanol was not critical and that extraction equilibrium was attained within 1 or 2 hours. The concentrations of subtilin obtained in the extracts were far below the solubility limits of subtilin. Thus, the assays should represent total subtilin of the cultures unless "bound" subtilin, unextractable under these conditions, occurs in the cultures. The generally higher assays found for cultures adjusted to pH 2.5 before extraction with ethanol (Tables II and III), together with other

TABLE III

*Rate of Extraction of Subtilin from Bacillus subtilis Culture<sup>1</sup> with 60% Ethanol*

pH of culture before extraction	Time of extraction				
	5 minutes	15	45	2 hrs.	22
	<i>mg of subtilin in the extract/l. of culture</i>				
7.1 (unadjusted)	410	420	430	480	440
4.0 (acetic acid)	400	420	450	460	450
2.5 (HCl)	420	510	540	540	540

<sup>1</sup> 44-hour 8% asparagus juice surface culture (same culture as in Table II); assayed with *M. conglomeratus*. Extraction volume 5 times original culture aliquot.

preliminary results, has stimulated investigation of the latter possibility. The results of such investigations will be presented later.

It was suggested previously (6) that cultures be prepared for assay by adjusting to pH 2.5 and autoclaving. Data obtained subsequently showed that activity was lost from steamed cultures (Table IV). Destruction occurred more rapidly at a pH near neutrality than at pH 4 or 2.5. Assays of the samples showing about 50% destruction by means of *S. faecalis* and *S. aureus* agreed with the results obtained with *M. conglomeratus*. It appears that the rate of destruction may vary from one type of material to another; of those tested, crude beet molasses culture was least stable, and partially purified standard subtilin was most stable. The difficulty of insuring quantitative retention of antibiotic activity during heat sterilization thus adds to the disadvantages of a growth-inhibition assay involving overnight incubation as compared with the short incubation-period assays where sterilization of samples is unnecessary.

*Antibiotic Heterogeneity of Subtilin Preparations.* It was suggested previously that subtilin is antibiotically heterogeneous (6). This has been confirmed by the following observations: (a) Different preparations gave dosage-response curves with different shapes or slopes; (b) the relative potencies of different preparations varied for the different test organisms; (c) mixtures of different preparations showed non-additive antibiotic activities; and (d) the course of inactivation by formaldehyde differed for different preparations.

TABLE IV  
Heat Lability of Subtilin in Crude Cultures

Sample and treatment	Initial subtilin content of sample <sup>1</sup>	Per cent of activity retained after holding for (minutes)			
		5	15	45	120
Standard subtilin (Lot 1263) in pH 2.5 HCl held at 98-99°C.:	40		90	98	
13-hr. supplemented asparagus juice submerged culture; aliquots held at 98-99°C.:	430 <sup>2</sup>				
harvest pH (6.4)		81	60	23	14
pH 4.0 with acetic acid		100	86	51	28
pH 2.5 with HCl		98	86	65	30
44-hr. asparagus juice surface culture (blended thoroughly); aliquots held at 98-100°C.:	520 <sup>2</sup>				
harvest pH (7.1)		96	90	52	23
pH 4.0 with acetic acid		90	96	69	42
pH 2.5 with HCl		100	96		71
44-hr. beet molasses surface culture (blended thoroughly); aliquots held at 104-106°C.:	720		(20 minutes)		
harvest pH (7.0)			29		
pH 1.8 with HCl			15 <sup>3</sup>		

<sup>1</sup> Assayed with *M. conglomeratus*. Concordant results were obtained with *S. aureus* and *S. faecalis*.

<sup>2</sup> All samples of these groups were adjusted to pH 2.5 with HCl before extraction with 60% ethanol.

<sup>3</sup> 10% of the original activity remained after the steamed sample had been autoclaved (115°C.) for an additional 20 minutes.

Two preparations with markedly divergent dosage-response curves are compared in Fig. 2. The relative potency of the two preparations varied with the fraction of total inhibition at which the comparison was made. If one such preparation was adopted as a standard and the other was assayed against it, a trend in assay value with size of aliquot was obtained. Such trends demonstrate an essential difference in the

character of the antibiotic agents, or of the presence of interfering factors. Trends representing dosage-response curves with greater and with less slope than standard subtilin have been found both with crude culture extracts and with partially purified preparations.

The relative responses given by the three test organisms to several different lots of partially purified subtilin are shown in Table V. Since 10% differences in potency are statistically significant, it is clear that the four lots of subtilin were antibiologically heterogeneous. Differences of similar and greater magnitudes have been observed frequently with

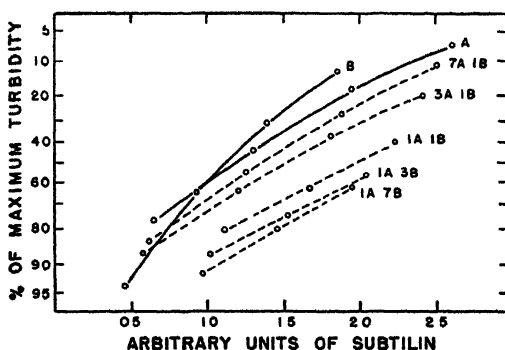


FIG. 2. Antagonistic interaction between two partially purified lots of subtilin on *Staphylococcus aureus*. The solid lines give the response to solution A (containing 3.06  $\gamma$ /ml. of lot L1254; see Table V) and to solution B (containing 0.80  $\gamma$  per ml. of lot L1242; see Table V). The broken lines give the response to mixtures of solutions A and B in the proportions indicated. The experimental points correspond to 1, 2, 3, and 4 aliquots (1.25 ml. each) of the solutions. The arbitrary unit of subtilin is that amount required to give 40% inhibition with the unmixed solutions; i.e., 7.83  $\gamma$  of L1254 (A) or 2.51  $\gamma$  L1242 (B).

crude culture extracts and with various fractions obtained during the preparation of subtilin.

The non-additivity of antibiotic effect of mixtures of two solutions of partially purified subtilin preparations is shown in Fig. 2. Solutions A (of subtilin lot L1254) and B (of subtilin lot L1242) were adjusted to possess approximately the same specific activity against *S. aureus*. The curves for mixtures of A and B fall to the right of the curves A and B for the unmixed solutions, instead of between A and B, as would be expected by interpolation, assuming there was no antagonism between



TABLE V  
*Relative Response of the Test Organisms to Different  
 Lots of Partially Purified Subtilin*

Subtilin lot	Relative potency by		
	<i>M. conglomeratus</i>	<i>S. faecalis</i>	<i>S. aureus</i>
L1263 (standard) <sup>1</sup>	per cent (100)	per cent (100)	per cent (100)
L1242 <sup>2</sup>	101	102	121
L1254 <sup>3</sup>	102	120	25 to 50 <sup>6</sup>
L1277 <sup>4</sup>	152	124	147

<sup>1</sup> Prepared by a precipitation-extraction method similar to the published method (3); the precipitate obtained on concentration of 70% ethanol extract of *Bacillus subtilis* pellicle was extracted with 95% ethanol, removing inactive materials and yielding "Fraction B" (3). Fraction B was not extracted with saline acidified 85% ethanol as described; instead it was dissolved in glacial acetic acid and diluted to 1% acetic acid. The soluble fraction was lyophilized to yield lot L1263 (the arbitrary standard).

<sup>2</sup> A composite of lots prepared by the foregoing procedure through Fraction B; the active material was recovered from Fraction B (in low yield) by dialysis against 1% acetic acid, followed by resin deionization and lyophilization to yield lot L1242.

<sup>3</sup> Prepared by an adsorption-elution procedure. Subtilin was adsorbed on a cation exchange resin (Duolite C-1) from the 70% ethanol extract of asparagus medium pellicle. After washing the column with 70% ethanol the subtilin was eluted with 70% ethanol containing 5% NH<sub>4</sub>Cl. The eluate was concentrated to remove ethanol whereupon the subtilin precipitated. The precipitate was washed with a small amount of 0.2 M acetate at pH 4.5 and dialyzed against 1% acetic acid. The diffusate was deionized with Amberlites IR-100 and IR-4, and lyophilized to give lot L1254.

<sup>4</sup> Prepared by the published (3) precipitation-extraction procedure.

<sup>6</sup> Marked assay trend; see Fig. 2.

the active materials of solutions A and B. The loss in potency can be accounted for as due entirely to inactivation of B in the presence of A, since the curves for the mixtures of 7A:1B and 3A:1B show activities which calculate correctly for the A component. The inactivation of A by B seems unlikely, since, for the mixture of 1A:7B, the extent of the loss exceeds considerably that expected were all of the A component inactivated. An extension of the experiment to wider ratios of A and B gave similar results. Low recoveries, frequently obtained when mixtures of standard and unknown preparations were assayed, suggest that the phenomenon may have influenced many of the subtilin assays made with *S. aureus*.

A difference in the course of inactivation of two classes of subtilin preparations by formaldehyde is shown in Table VI. The higher potency preparations, of which L1113 was typical, apparently were much more sensitive to formaldehyde than was the lower potency preparation

TABLE VI  
*Lability of Partially Purified Subtilin to Formaldehyde<sup>1</sup>*

Subtilin lot	Concentration of formaldehyde (per cent)			
	0.00 (control)	0.01	0.1	1
L1113 <sup>2</sup>	(100)	percentage 64	residual activity 26	21
L1254	40 (marked trend)	35 (marked trend)	21 (slight trend)	30 (no trend)

<sup>1</sup> Subtilin solutions (1 mg. of solids/ml.) in 70% ethanol containing the concentrations of formaldehyde indicated were held at about 4°C. for 48 hours at which time they were diluted with pH 2.5 HCl for immediate assay (*S. aureus*). The assays were calculated relative to the formaldehyde-free control of L1113.

<sup>2</sup> This lot of subtilin (and two similar lots which gave almost identical data for formaldehyde inactivation) was prepared by the method described in Table V, footnote 2, for lot L1242.

<sup>3</sup> See Table V, footnote 3, for method of preparation.

L1254. Formaldehyde treatment resulted not only in loss of potency, but also in a change in the character of the antibiotic activity of L1254, as is demonstrated by the disappearance of the marked assay trend given by untreated L1254.

Certain of the observations on antibiotic heterogeneity of subtilin lot L1254, as compared with standard subtilin lot L1263 or similar lots, may be correlated by assuming that lot L1254 contains an antagonist of subtilin activity for *S. aureus* which is without effect on subtilin activity for the other two test organisms. This would account for the similar potencies of L1263, L1242, and L1254 (Table V) when assayed by *M. conglomeratus* and *S. faecalis*, compared to the low potency and trends found with L1254 when *S. aureus* was used as the assay organism. The non-additivity of response mixtures of L1242 and L1254 would be accounted for by counteraction of the antibiotic activity of L1242 by the antagonist in L1254. In the experiment on the treatment of subtilin with formaldehyde (Table VI), the course of inactivation of the antibiotic agent may be quantitatively similar for both lots of subtilin, while the apparently divergent courses of inactivation of antibiotic activity and the alteration in the shape of the dosage-response curve (elimination of assay trends) may be related to inactivation of the subtilin antagonist. The possibility that the antagonist

may be a growth factor required by *S. aureus*, but not by the other two assay organisms, has not been investigated specifically. The known vitamins cited in the next paragraph were inactive, however.

*Effect of Various Compounds on the Activity of Subtilin.* A number of amino acids, vitamins, and related compounds were tested for effects on the bacteriostatic action of subtilin against the three assay organisms. They were tested with levels of subtilin sufficient to give about 50% inhibition under the conditions of the assay method. Included at levels up to 16 mg. per tube were alanine, arginine, aspartic acid, asparagine, cysteine, glutamic acid, glutamine, glycine, histidine, homocysteine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine, an acid hydrolyzate of casein (Difco casamino acids), adenine, and uracil. Thiamin, riboflavin, nicotinic acid, nicotinamide, pantothenic acid, pyridoxine, pyridoxal, pyridoxamine, *p*-aminobenzoic acid, choline, inositol, and  $\beta$ -alanine were tested at levels up to 4 mg. per tube. Synthetic biotin and folic acid (Folvite) were tested at levels up to 40  $\gamma$  per tube. Most of the compounds had no effects, but those that did gave similar effects in cultures containing subtilin and control cultures without subtilin. None of the compounds would be expected to occur in assay samples in amounts sufficient to affect the assay of subtilin.

## DISCUSSION

The investigations in which the method described in this paper were used had the limited objectives of developing methods for the ready production of *Bacillus subtilis* cultures containing a good level of antibiotic activity (1, 2), and of developing a method for the isolation of antibiotic material of high specific activity and low toxicity (3). A single assay organism was used for routine quantitative data on samples which could be assumed to be antibiotically similar. The latter assumption could then be partially confirmed by parallel assays with the other test organisms whenever a new cultural medium was introduced or whenever a new fractionation step was introduced in the purification procedure. This was considered advisable after the adsorption-elution procedure used to prepare lot L1254 (see footnote 3, Table V) was abandoned for a precipitation-extraction procedure on the basis of greatly increased potency for *S. aureus*, the only organism used for assays at the time. Subsequent comparisons with other organisms (see

Table V) gave similar potencies for subtilin preparations by the two procedures, suggesting that the new procedure had not achieved markedly greater concentration of the actual antibiotic, but had only resulted in the elimination of a subtilin antagonist particularly effective with *S. aureus*.

In general, however, favorable treatments were consistently so by all the test organisms, and comparisons by the several test organisms have rarely disagreed by as much as 50% of the smaller assay value. Thus it was possible to defer the question of specific assays for components of a hypothetical "subtilin complex" until subtilin (or a member of the complex) could be obtained in pure form and some decision made as to whether the heterogeneity under discussion is due to a group of chemically similar antibiotics or to a single antibiotic and secondary synergistic and antagonistic factors. In view of the interactions demonstrated between different lots of subtilin, it would seem that specific assays will be dependent on actual separation or on differential inactivation of the different components of the subtilin antibiotic complex.

Early experience with subtilin prepared by adsorption showed that different lots varied widely in the fraction of the total antibiotic activity of resin eluates which would diffuse through cellophane (3). In particular, as little as 10 or 15% was diffusible in the case of certain concentrates from microbial production batches characterized by good growth but relatively low antibiotic activity. It appeared that the normally diffusible antibiotic was being adsorbed by chemically similar, less readily diffusible, antibiotically inert substances. These considerations caused us to avoid a plate assay for subtilin. Actual comparisons by turbidimetric and plate methods have not been made, however.

#### ACKNOWLEDGMENT

We wish to acknowledge our indebtedness to Dr. T. H. Jukes of the Lederle Laboratories for synthetic folic acid.

#### SUMMARY

A turbidimetric assay method for subtilin is described which depends on growth inhibition of *Micrococcus conglomeratus*, *Streptococcus faecalis*, or *Staphylococcus aureus*. The cultures were incubated for 4 or 5 hours at 37°C. under non-sterile conditions. Additional evidence is presented

that the antibiotic activity of crude culture extracts of *Bacillus subtilis* and of partially purified lots of subtilin is not homogeneous. The activity of subtilin under assay conditions was unaffected by various compounds, including the common amino acids and vitamins of the B group.

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# The Chemistry of Infectious Diseases. VIII. Partial Amino Acid Composition of Purified Dog Serum Albumins before and during Type I Pneumococcal Pneumonia<sup>1</sup>

Bruno Vassel<sup>2</sup>, Ruth Partridge and M. L. Crossley

*From the Stamford Research Laboratories, American Cyanamid Company,  
Stamford, Connecticut*

Received June 3, 1947

## INTRODUCTION

The constancy of the cystine content of dog serum albumins during experimental Type I pneumococcal pneumonia was questioned in one of the previous papers in this series (1). The data indicated that a decrease occurred in the cystine concentration of the serum albumins during the course of the infection, while there was no change in cystine content of the serum globulins. These conclusions were the results of cystine analyses of once-precipitated albumins isolated by means of one of the conventional, clinical methods of albumin/globulin fractionations (2), a method primarily devised to follow changes routinely in albumin/globulin ratios. The validity of our previous conclusions, therefore, depended greatly on the relative purity of the albumins isolated from the blood of normal dogs, and of dogs during the infection.

Leutscher (3) recently compared plasma salting-out methods with electrophoretic measurements. He concluded that "the albumin/globulin ratio (from salt precipitations) is reasonably accurate so long as the albumin is not greatly reduced, and so long as globulin increase does not involve the fractions ( $\beta$ -globulins) which seriously contaminate the albumin fraction." The two limitations stipulated by Leutscher are precisely those which one would expect to encounter during inflammatory, febrile pneumonia.

<sup>1</sup> Dedicated to Professor Carl Neuberg on his 70th birthday.

<sup>2</sup> Present address, Amino Products Division, International Minerals and Chemical Corp., Rossford, Ohio.

The mean plasma volume is known to increase significantly during pneumonia, and to decrease to levels below normal immediately following recovery (4). This change in plasma volume affects adversely the constancy of plasma protein precipitations by salts (5). Furthermore, an increase in the plasma lipid content seems to exert an increasing solubilizing effect on plasma proteins (6), which is exactly what one encounters in pneumonia, where the absolute amounts of circulating, lipid-rich  $\alpha$ -globulins are at a high level (7, 8).

Consequently, it was deemed advisable to isolate more highly purified albumin preparations from normal dogs, and from dogs at the height of infections, and to redetermine their cystine contents. No data on the amino acid composition of purified dog serum albumins have been published. Therefore, as many amino acids other than cystine were also determined, as micro methods and protein material were available.

### EXPERIMENTAL

The care of the dogs, and their infection with Type 1 pneumococci <sup>2a</sup> has been described previously (9). The animals were bled to death under nembutal (1 ml./10 lb.) by canulating the jugular vein. The serum was collected in the customary manner by centrifugation after clot formation. The serum of the infected dogs was classified into 2 groups, serum from dogs with mild (A) and with severe (B) infections. This classification, admittedly arbitrary, depended primarily upon an evaluation of the physical appearance of the animal, and on the degree of consolidation of the lungs just prior to death, as shown by X-rays. Fever is not a reliable criterion of the severity of the infection (9).

Totals of 4890 cc. of normal, 2800 cc. of mildly infected-A, and 1600 cc. of severely infected-B sera were collected. The greatest possible care was exercised to treat all 3 sera as nearly alike as possible during the salt fractionations, in order to obtain an approximation of the relative amounts of each protein fraction of each of the sera. After dilution with an equal volume of water, saturated ammonium sulfate solution was added to remove the various globulin fractions until 50% saturation was reached at pH 6.8-6.9.<sup>3</sup> The supernatants were then further fractionated in accordance with Hewitt's procedure (10, 11) into crystalbumin (insoluble in 50% saturated ammonium sulfate at pH 6.8 at the first precipitation, but soluble at all subsequent precipitations at 50% salt saturation at pH 6.8, but not at pH 4.7), globoglycoid (insoluble at the first and all subsequent precipitations in 50% saturated ammonium sulfate at pH 6.8), seroglycoid (insoluble in 66% saturated ammonium sulfate at pH 5.0), and proteoses which settled upon full salt saturation of the supernatant from the sero-

<sup>2a</sup> We gratefully acknowledge the valuable cooperation we received from Mr. Robert J. Fitzgerald in the preparation of the pneumococci cultures, and the inoculations of the dogs.

<sup>3</sup> Details of the globulin fractionation will be presented in Paper IX of this series.

glycoid precipitation at pH 6.8. The globoglycoids of the 3 types of sera dissolved with increasing difficulty at pH 6.8 in water after each new reprecipitation. It finally became necessary to raise the pH to 7.5 to effect solution. These proteins were very slimy, and greenish in color. The crystalalbumin isolated from the infected-B serum had a distinct red tinge, not noted in the other 2 preparations.

Each fraction was reprecipitated 5 or 6 times, except for the proteoses, which appeared to be free of other proteins after 3 precipitations. The proteins were dialyzed in rotating Visking casings against distilled water at 34°F. until negative sulfate tests were obtained. The proteins were then precipitated with 5 volumes of cold acetone, dried *in vacuo* at room temperature over  $P_2O_5$ , ground, and dried again. The proteoses, because of small yields, were not dialyzed. Total nitrogen was determined by micro Kjeldahl (12); sulfur by the Pregl method; sulfate-sulfur gravimetrically by Folin's (13) method; ash by heating at 550°C. for 12 hours; total lipids by petroleum ether extraction in a Soxhlet, and weighing of the lipids after evaporation of the solvent *in vacuo* and drying over  $P_2O_5$ ; carbohydrates by Hewitt's (11) adaptation of Sørensen and Haugaard's (14) method, using an equal molar mixture of galactose-mannose as the arbitrary reference solution, with a correction for the probable presence of hexoseamine, assuming 1 mole of hexoseamine for each 2 moles of galactose-mannose.

For tyrosine and tryptophan analyses, 100-200 mg. of proteins were hydrolyzed with 2 cc. of 5 *N* NaOH at 100°C. for 16 hours in sealed, almost completely filled glass vials. The hydrolyzates were filtered through sintered glass, acidified with 1 cc. of 20 *N*  $H_2SO_4$ , and diluted with water. Aliquots were analyzed by Lugg's method (15) at 4900 and 4250 Å for tyrosine and tryptophan, respectively. Tyrosine values were corrected by Brand's (16) 1.044 factor. Some of the tryptophan analyses were checked by Dr. Lampen of this laboratory by his microbiological method.<sup>4</sup> Agreement in all cases was excellent.

Methionine was determined by McCarthy and Sullivan's nitroprusside method (17) on proteins hydrolyzed with 4 cc. of 5 *N* HCl at 125°C. for 10 hours. The presence of ammonium sulfate, in quantities such as one encounters in ammonium sulfate precipitated, undialyzed proteins (proteoses), depressed the color slightly. The presence of only traces of iron in the decolorizing charcoal caused serious methionine losses from the hydrolyzates. McCarthy and Sullivan recommended for decolorization Carboraffin (Carbex B), a preparation now commercially unavailable. Norit, after exhaustive washings with hot, dilute HCl until the filtrates gave a negative potassium thiocyanate test, proved satisfactory.<sup>5</sup> After the completion of the analyses, White and Koch's note (18) came to our attention in which they reported a depressing effect of cystine on the methionine-nitroprusside color, if the cystine/methionine ratio was high. The  $-S-S-$  +  $-SH$  to methionine ratio of our normal crystalalbumin preparation had a ratio of  $6.5/1.2 = 5.4$  (Table III), a ratio similar to the one reported by White and Koch as causing low methionine values. It appears, however, that our methionine values are, nevertheless, close to true values, for the calculated sum of cystine, cysteine and methionine sulfur agrees closely with the analytical values for sulfur by the Pregl method (Table V).

<sup>4</sup> Lampen, J. O., Unpublished method.

<sup>5</sup> Upon Dr. M. X. Sullivan's suggestion, we investigated the possibility that iron was the interfering substance in our charcoal.



For cysteine and cystine analyses the proteins were hydrolyzed under an atmosphere of nitrogen with formic acid-hydrochloric acid, and analyzed by Vassel's *p*-dimethylaminoaniline method (19) with no changes from the published procedure. High ammonium sulfate concentrations in the proteoses had no effect on the color.

Serine and threonine determinations were performed on the proteins after hydrolyses with 5 cc. of 6 *N* HCl in the presence of 0.5 g. of urea at 125°C. for 16-18 hours. The hydrolyzates were treated with Darco, filtered through sintered glass, and made to volume with water. Both amino acids were determined by Nicolet and Shinn's (20, 21) methods, with the following modifications: the bisulfite solution recommended by Troy and Sharp (22) was substituted, and the bound bisulfite was liberated with sodium carbonate buffer recommended by Goldman and Yagoda (23) instead of with dry sodium bicarbonate. Serine was weighed as the dimedon derivative, but no solubility correction was applied.

Histidine, arginine, and lysine were determined by Block's (24) microadaptation of the Kossel procedure. Because 2.5 g. of protein for each analysis is the recommended amount, only the crystalbumins were analyzed (in duplicate) for the basic amino acids. Histidine was weighed as the nitrilate; arginine as the flavianate and corrected by the Gulewitsch factor (25) of 0.036 g./l. for the solubility of the arginine silver in the presence of excess barium hydroxide; lysine as the picrate with a correction factor of 10 mg. for the lysine picrate.

The absence of hydroxyproline from any of the protein preparations was established by the use of McFarlane and Guest's (26) oxidative-colorimetric method on 100 mg. of protein samples, hydrolyzed with 5 cc. of 6 *N* HCl at 115-125°C. for 18 hours.

## RESULTS

The isolation, separation, and purification of the components of a mixture of proteins by salt and isoelectric precipitation can at best be only semi-quantitative. If larger differences in the amounts of isolated proteins are encountered than can reasonably be expected to result from manipulative errors, it should be permissible to consider such differences as indicative of the existence of certain trends. With these considerations in mind, an inspection of Table I indicates that the crystalbumin fraction remained essentially constant, that the globoglycoid, proteose, and globulin fractions showed a tendency to increase, and the seroglycoid fraction a tendency to decrease from normal values during the infection. The constancy of the crystalbumin fraction may, at first, seem surprising, and contrary to the accepted axiom that febrile and severe inflammatory diseases decrease the amounts of circulating serum albumins. A possible explanation of this apparent discrepancy is discussed below. The increase in the globulin concentration is consistent with the well known behavior of these proteins during febrile, inflammatory diseases, as is also the increase in the globoglycoid

TABLE I

*Distribution of the Serum Protein Fractions in Each 100 g.  
of Isolated Total Serum Proteins*

All values are corrected for moisture, ash, lipids and carbohydrates

	Dog serum from		
	Normal	Infected-A	Infected-B
	<i>g.</i>	<i>g.</i>	<i>g.</i>
Crystallbumin	48.41	46.81	49.12
Globoglycoid	5.31	1.62 <sup>1</sup>	6.73
Seroglycoid	7.80	6.45	1.83
Proteoses	0.77	1.73	1.96
All globulins	37.71	43.41	40.37

<sup>1</sup> Low value due to accidental loss during the purifications, consequently, each of the other protein fractions of the Infected-A serum should in reality be lowered by an estimated 4-5% of the given value.

fraction which, according to Svensson's electrophoretic data (27) is in reality a mixture of  $\alpha$ - and  $\beta$ -globulins which had remained in solution at 50% ammonium sulfate saturation. Our data in Table I are, therefore, not at variance with Leutscher's (3) analyses of certain pathological sera, even though he had not actually studied blood of pneumonia patients. Also Taylor and Keys (28) note that the fractionation of pathological serum agrees less well with electrophoretic data than does normal. It therefore appears quite certain that our previously analyzed, once-precipitated "albumins" of infected dogs of Paper VI of this series (1) were grossly contaminated with larger amounts of globulins than were the "albumins" of the normal dog sera. Table I also shows a very pronounced drop in the seroglycoid fraction of the sera from infected animals.<sup>6</sup> Since this fraction is always a part of the "albumins" reported in conventional albumin/globulin fractionation procedures, its marked decrease during infection may, in part at least, account for the often reported decreases in total circulating albumins during inflammatory diseases. Hence, the drop in seroglycoid concentration may be the explanation of our finding that little, if any, changes occur in the amounts of the true crystallbumins, yet an overall decrease in total

<sup>6</sup> We feel quite confident that the 1.82 g. value for the seroglycoid fraction of the infected-B serum is approximately correct, and not low primarily due to abnormally large losses during its isolation.

albumins occurs. Electrophoretic data, until recently, have given no evidence that at neutral or slightly alkaline pH ranges the serum albumin fraction contained more than one component (29, 30). This would invalidate our discussion of crystalbumin and seroglycoid, were it not for the recent publications of Blix (7), Luetscher (31), and perhaps Blix, Tiselius, and Svensson (8), who observed 2 distinct boundaries in serum albumins provided that electrophoresis was continued for a much longer time interval and at a lower pH than had hitherto been the practice.

Of all the values presented in Table I, those of the proteoses are the least subject to manipulative errors. The proteoses show little tendency to coprecipitate with the other proteins at salt concentrations below saturation, but precipitate readily at full saturation. The sera of the infected dogs contained, respectively, 2.25- and 2.5-times as much proteoses as the normal sera. This is in excellent agreement with our previous serum-peptone-nitrogen and polarographic observations where increases in peptone-nitrogen and polarographic peptone wave height of 100% and more was reported (9). This increase in the small molecular weight protein fraction of blood serum during pneumonia—and very likely in other deep-seated inflammatory diseases—undoubtedly is indicative of some profound metabolic disarrangement, on which little research has as yet been done.

The nitrogen, sulfur, carbohydrate, and lipid contents of 3 of the serum proteins under discussion are given in Table II. No sulfur values for the proteoses are given in Table II; to give them would require a relatively very large correction for the ammonium sulfate content of these undialyzed fractions which would make the calculated values of questionable significance. Except for the globoglycoid, whose nitrogen content increases to a greater extent that can be accounted for by analytical errors, the other 3 fractions show no differences between normal and infected values. The increase in the nitrogen content of the globoglycoids is not due to a higher lipid content of the normal proteins since all nitrogen values in Table II have been calculated on a lipid (and ash, moisture, and carbohydrate)-free basis. The sulfur contents of the 3 crystalbumins are constant. The increase in sulfur concentration in the globoglycoids and the decrease in the seroglycoids is reflected in their cystine concentrations, which will be discussed below. The carbohydrate of the crystalbumins is essentially constant, and the

TABLE II

*The Nitrogen, Sulfur, Carbohydrate, and Lipid Content of Purified Crystalbumin, Globoglycoid, Seroglycoid, and Proteoses of Normal and of Type I Pneumococcal Infected Dog Sera*

All values are corrected for moisture and ash

	Crystalbumin			Globoglycoid			Seroglycoid			Proteoses		
	Normal	Infected		Normal	Infected		Normal	Infected		Normal	Infected	
		A	B		A	B		A	B		A	B
Per cent nitrogen <sup>a</sup>	14.8	14.0	14.6	12.2	13.7	14.0	14.9	15.1	14.7	16.5	15.3	16.0
Per cent sulfur <sup>a</sup>	2.3	2.3	2.3	0.9	1.25	1.3	1.95	1.7	1.3	6	6	6
Per cent carbohydrate	1.0	1.0	1.3	8.7	6.1	6.3	5.7	6.4	6.4 <sup>c</sup>	26.8	26.2	24.4
Per cent lipid	1.3	0.34	0.33	13.2	3.5	6.5	0.55	1.0	2.8	1.2	1.4	1.2

<sup>a</sup> Calculated on ash-, moisture-, carbohydrate-, and lipid-free basis.

<sup>b</sup> The presence of large amounts of ammonium sulfate, relatively very small amounts of protein material, and a small yield of the undialyzed dry "proteose" made the estimation of protein-sulfur a fruitless undertaking.

<sup>c</sup> Estimated value due to insufficient amount of protein.

variation from the normal value in cases of the globoglycoids, seroglycoids and proteoses is of questionable significance.

There appears to be a marked decrease in lipid concentration from the normal in the infected sera of both the crystalbumins and the globoglycoids, and an increase in the infected seroglycoids. The relatively high lipid values of the globoglycoids are consistent with the claim that they are in reality the lipid-rich  $\alpha$ - and  $\beta$ -globulins (27), but the decrease in their lipid content during infection was unexpected. No significant differences in the lipid contents of the proteoses were observed.

*Amino Acid Composition.* The amino acid composition of the 4 proteins, corrected for ash, moisture, carbohydrate, and lipids, is given in Table III, together with the estimated probable percentage errors of each analytical method. These latter values represent the maximum variations from the mean of 10 analyses of each amino acid. For the purpose of comparing the analytical amino acid values of normal and of infected dog serum albumins, Block and Bolling's (24) suggestion was adopted, which calls for the recalculation of all values to a 16% protein nitrogen content. It is a logical correction for those unknown factors which cause differences in the nitrogen concentration of supposedly the same kind of proteins. The recalculated amino acid composition is

TABLE III

*Amino Acid Composition of Crystalbumin, Globogloboid, Serogloboid, and Proteose of Normal Dog Serum, and of Dog Serum During Mild (A), and Severe (B) Pneumococcal Pneumonia*

All values are calculated on ash-, moisture-, carbohydrate-, and lipid-free proteins

	Crystalbumin			Globogloboid			Serogloboid			Proteose		
	Normal	Infected		Normal	Infected		Normal	Infected		Normal	Infected	
		A	B		A	B		A	B		A	B
	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
Methionine	1.2	1.2	1.5	1.9	2.0	2.0	2.4	2.3	—	2.5	2.1	2.4
Cysteine	1.1	1.2	1.3	1.1	1.4	1.1	1.5	1.3	1.9	1.0	0.7	0.7
Cystine	5.4	5.1	4.8	1.2	2.2	2.5	3.3	2.9	2.0	1.2	0.8	1.0
Cystine+cysteine	6.5	6.3	6.1	2.3	3.6	3.6	1.8	4.2	3.9	2.2	1.5	1.7
Tyrosine	6.1	5.9	5.1	5.0	6.0	5.3	6.3	6.2	6.05	5.4	4.7	5.8
Tryptophan	0.5	0.4	0.3	0.9	1.5	0.6	2.1	1.5	2.0	0.8	1.0	0.9
Serine	8.4	6.9	7.2	19.1	14.5	12.3	11.2	8.6	18.5	20.2	18.6	12.5
Threonine	10.3	6.1	5.4	6.8	10.7	10.1	9.0	9.1	15.3	12.1	9.5	6.1
Hydroxyproline	0	0	0	0	0	0	0	0	0	0	0	0
Arginine	5.5	5.1	5.4									
Histidine	2.6	2.2	1.8									
Lysine	4.9	7.2	6.9									

given in Table IV; it will be used exclusively for the discussion of the results.

*Crystalbumin.* The methionine, cysteine, cystine, tryptophan, serine and arginine contents of the 3 isolated crystalbumins are constant; these values all fall within the probable errors of the methods. A decrease in tyrosine, threonine and histidine and an increase in lysine concentrations seems indicated from the data of the infected animals. No attempt will be made at this time to interpret these and any of the other differences in amino acid compositions of the 4 isolated protein fractions until the isolations and amino acid analyses have been repeated on a new set of normal and infected dogs. It is our intention to merely call attention to those differences in the amino acid composition of the 4 proteins which appear to be larger than one would expect merely as a result of analytical errors.

*Globogloboids.* The methionine, cysteine, tyrosine, tryptophan, and threonine values indicate no variations within the 3 globogloboids, but

an increase in cystine (hence also in the total  $-SH + -S-S-$ ), and a pronounced decrease in serine content is indicated. As will be shown in a subsequent report, the observed decrease in serine concentration is not paralleled by a similar decrease in the serine content of the euglobulins or pseudoglobulins, although the globoglycoids are thought to be unprecipitated serum globulins (27, 32).

TABLE IV

*Amino Acid Composition of Crystallalbumin, Globoglycoid, Seroglycoid, and Protease of Normal Dog Serum, and of Dog Serum During Mild (A), and Severe (B) Pneumococcal Pneumonia*

All values are calculated on a 16% protein nitrogen content

	$\pm$ error <sup>a</sup>	Crystallalbumin			Globoglycoid			Seroglycoid			Protease		
		Normal		Infected	Normal		Infected	Normal		Infected	Normal		Infected
		Per cent	A	B	Per cent	A	B	Per cent	A	B	Per cent	A	B
Methionine	6.6	1.3	1.3	1.6	2.5	2.3	2.3	2.6	2.4		2.1	2.5	2.4
Cysteine	3.0	1.2	1.3	1.4	1.4	1.6	1.3	1.6	1.1	2.0	0.9	0.7	0.7
Cystine	c	5.8	5.5	5.3	1.6	2.6	2.9	3.5	3.1	2.1	1.2	0.8	1.0
Cystine + cysteine	3.0	7.0	6.8	6.7	3.0	4.2	4.2	5.1	4.5	4.1	2.1	1.5	1.7
Tyrosine	2.0	6.6	6.3	5.9	6.6	7.0	6.1	6.8	6.6	6.3	5.2	4.9	5.8
Tryptophan	2.8	0.5	0.4	0.3	1.2	1.8	0.7	2.3	1.6	2.1	0.8	1.0	0.9
Serine	13.2	9.1	7.4	7.9	25.0	16.9	14.1	12.0	9.1	19.2	19.6	19.2	12.5
Threonine	13.8	11.1	6.6	5.9	8.9	12.5	11.5	9.7	9.6	15.9	11.7	9.8	6.1
Hydroxyproline		0	0	0	0	0	0	0	0	0	0	0	0
Arginine	5.8 <sup>b</sup>	5.9	5.5	5.9									
Histidine	7.6 <sup>b</sup>	2.8	2.1	2.0									
Lysine	9.8 <sup>b</sup>	5.3	7.7	7.6									

<sup>a</sup> The numbers given in this column represent maximum deviations from mean of 10 analyses.

<sup>b</sup> Calculated from the mean of duplicate analyses only.

<sup>c</sup> Cystine = (cysteine + cystine) - cysteine.

*Seroglycoids.* No variations in the methionine, cysteine, and tryptophan values from those of the normal animals were observed. Small decreases in the cystine (and  $-SH + -S-S-$ ) and, perhaps, tyrosine concentrations during infection seem to take place. The analytical values for serine and threonine, repeated many times, were unexplainably erratic. Table IV presents those serine and threonine values which

we believe are the best, but they are, nevertheless, of questionable significance. Our results are in agreement with Hewitt (11), who found that seroglycoid has a higher tryptophan and a lower cystine content than crystalbumins.

*Proteoses.* The methionine, tyrosine, tryptophan, and probably serine contents, of the 3 proteoses are constant. There appears to be a slight, probably significant decrease in cysteine, cystine, and perhaps threonine concentrations in the infected animals.

As a check on the accuracy of the determinations of the sulfur-containing amino acids, the total sulfur contents of the crystalbumin, globoglycoids and seroglycoids, calculated from the sum of the analytical cysteine, cystine, and methionine values, are compared in Table V with the analytical Pregl total-sulfur analyses. It is evident that good

TABLE V  
*Pregl Sulfur-Content of Normal, Mildly Infected-A, and Severely Infected-B Dog Serum Crystalbumins, Globoglycoids, and Seroglycoids, Compared to Calculated Sulfur Values from the Sum of the Cysteine, Cystine, and Methionine Analyses*

	Crystalbumin			Seroglycoid			Globoglycoid		
	Normal	Infected-A	Infected-B	Normal	Infected-A	Infected-B	Normal	Infected-A	Infected-B
	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
Sum of cysteine, cystine, and methionine sulfur	1.99±0.07	1.94±0.07	1.94±0.07	1.80±0.07	1.60±0.06	1.52±0.07	1.02±0.05	1.39±0.06	1.39±0.06
Pregl sulfur	2.3±0.3	2.3±0.3	2.3±0.3	1.95±0.3	1.7±0.3	1.35±0.3	0.9±0.3	1.25±0.3	1.3±0.3

agreement exists between these figures, which enhances the value of those variations from normal values which have been discussed previously.

## DISCUSSION

We do not feel ready at this time to attach undue significance to any of the observed, and previously discussed, differences in the amino acid composition of normal and infected dog albumins. However, it is our desire to call attention to one observation which, in fact, was the original purpose of this investigation. It is the question as to whether or not the total cystine (+ cysteine) content of dog sera decreases during infection. Table VI presents the calculated cystine + cysteine content

TABLE VI

*Comparison of the Normal, Mildly Infected, and Severely Infected Dog Serum "Albumin"-Cystine Values Previously Reported on 4 Individual Dogs (1), and the Data on Pooled Dog Sera of This Paper*

	Per cent -S-S- + -SH of total serum albumin		
	Normal (average) <sup>1</sup>	Mildly infected <sup>1</sup>	Severely infected <sup>1</sup>
Dog 20	4.0	3.4	3.2
Dog 30	4.2	3.5	3.0
Dog 31	4.1	3.3	2.8
Dog 51	3.9	3.0	2.6
Pooled sera (see text)	3.66	3.32	3.33

\* See text for explanation of the numerical values.

of the "albumins" of each of the 3 types of dog sera. In making these calculations the per cent concentration of each of the 4 "albumins" (Table I)<sup>7</sup> was multiplied by the respective -S-S- + -SH percentages of Table III (*i.e.*, the amino acid composition *not* corrected to a 16% nitrogen content), and the numerical values obtained were added to yield total cystine + cysteine concentrations of each of the 3 serum "albumins." The decrease from 3.66% of the normal "albumin"-cystine content to 3.32% cystine for the mildly infected, and 3.33% for the severely infected animals is undoubtedly significant, especially in view of the good agreement between the calculated total sulfur, and the Pregl total sulfur values of Table V. For purpose of comparison the total "albumin"-cystine values of the 4 dogs reported in paper VI of this series (1) are given, the following assumptions being made: that the "normal" range of 3.9-4.2% albumin-cystine ((1), Fig. 3, p. 411) represents the mean of all the normal values of each dog; that the range of 3.0-3.5% cystine represents the mean values for each of the 4 dogs between normal cystine values and the lowest cystine value at the height of the infection; and that the range of 2.6-3.2% cystine gives the lowest values for each dog at the height of infection. Inasmuch as we are now comparing data on pooled sera, with data collected previously on individual dogs, a less pronounced spread in the values of the

<sup>7</sup> It was necessary to correct for the loss in the isolation of the globoglycoid of infected serum-A. A value of 6.0 g. protein was assigned, a value which lies above that of the normal dog serum (5.34 g.) but below that of the severely infected-B serum (6.73 g.). The other infected-A fractions were consequently reduced by 4.5%.



pooled sera must be expected; only when one is working with individual dogs can one hope to obtain blood at its lowest possible cystine content. Hence, the values of 3.66, 3.32, and 3.33 essentially confirm our previous findings on individual dogs of 3.9-4.5, 3.0-3.5, and 2.6-3.2, respectively, for normal, mildly, and severely infected dog serum albumin-cystine. Consequently, it appears that an actual decrease in total cystine content occurs during the infection.

Inspection of Table IV also reveals that the main decrease in cystine concentration of the "albumin" fraction occurs in the seroglycoids, and that this decrease is somewhat compensated by increases in the absolute amounts as well as in the cystine concentrations of the globoglycoids.

A comparison of our amino acid data with published values is not possible for two reasons: first, no data on dog serum albumins are available, and secondly, published amino acid values on human, horse, cattle, *etc.*, serum albumins do not differentiate between the 4 "albumin" fractions. Consequently, a recalculation of our data would be necessary, based on protein yield and amino acid composition, similar to the one attempted in Table VI. Since the yield data are admittedly only an approximation, no gain could be seen in such a calculation.

#### ACKNOWLEDGMENT

We wish to express our appreciation to Miss Suzanne Spitz for her technical assistance during a part of this investigation.

#### SUMMARY

Pooled sera of normal dogs, of dogs with mild, and with severe experimental Type I pneumococcal pneumonias were fractionated repeatedly with ammonium sulfate into reasonably pure crystalalbumins, globoglycoids, seroglycoids, and proteoses. Nitrogen, sulfur, carbohydrate, and lipid contents of each of the fractions are presented. The cystine, cysteine, methionine, tyrosine, tryptophan, serine, threonine, hydroxyproline, and in the case of the crystalalbumin, also the arginine, histidine, and lysine concentrations of each of the 3 types of sera are given. Certain trends of some of these amino acids toward increase or decrease during the course of the infection were found. An extended discussion of an observed decrease in the sum of the total cystine plus cysteine contents of the 4 "albumin" fractions is presented.

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# The Chemistry of Infectious Diseases. IX. Partial Amino Acid Composition of Salt-Fractionated Dog Serum Globulins before and during Type I Pneumococcal Pneumonia <sup>1</sup>

Bruno Vassel,<sup>2</sup> Ruth Partridge and M. L. Crossley

*From the Stamford Research Laboratories, American Cyanamid Company,  
Stamford, Connecticut*

Received June 3, 1947

## INTRODUCTION

Some amino acid analyses of 4 salt-fractionated serum albumins of normal dogs and of dogs during experimental Type I pneumococcal pneumonia were reported in the preceding paper in this series (1). The analyses indicated that during the infection the amino acid composition of the 4 albumins remained constant with respect to some of the amino acids, but that some of the others varied from the values of the normal amino acid concentrations to a greater extent than could be accounted for by purely analytical errors of the methods. The values for cystine plus cysteine essentially confirmed our previous observation (2) that the amounts of circulating  $-S-S- + -SH$  in the crude albumin fractions of once-precipitated serum albumins decreased during experimental lobar pneumonia in dogs.

It had also been shown previously (2) that the sum of the crude, once-precipitated serum globulins remained constant in their cystine plus cysteine contents during the course of the infection. Whether other amino acids in the serum globulins also remained unchanged during febrile diseases is not yet firmly established. The availability of sufficient amounts of crude normal and infected serum globulins for salt fractionations, and the lack of data on the amino acid composition of normal dog serum globulins appeared to warrant an investigation of

<sup>1</sup> Dedicated to Professor Carl Neuberg on his 70th birthday.

<sup>2</sup> Present address, Amino Products Division, International Minerals and Chemical Corporation, Rossford, Ohio.

their amino acid composition. The globulins were fractionated with ammonium sulfate into euglobulins, pseudoglobulins-I, and pseudoglobulins-II. The following amino acids were determined: cystine, cysteine, methionine, tyrosine, tryptophan, serine, threonine, and hydroxyproline.

### EXPERIMENTAL

The care of the dogs, their lobar infection with Type I pneumococci (3),<sup>2a</sup> and the collection of the sera have been described (1). The sera of the infected dogs were again divided into 2 groups: those from animals with mild infections (A), and those with severe infections (B), according to criteria previously discussed (1).

After the initial separation of the euglobulins at  $\frac{1}{2}$  salt saturation and pII 5.9, and of the albumins from the pseudoglobulins by half saturation of the diluted sera with ammonium sulfate at pII 6.8-6.9, the globulins were further fractionated with salt. Great care was again exercised to treat the 3 types of sera as much alike as possible during the various fractionation steps in order to obtain an approximation of the relative amounts of each of the globulins. The precipitated euglobulins were dissolved, 4 times in all, in 3% sodium chloride, an insoluble greenish slime filtered off, and the euglobulins reprecipitated at 32% ammonium sulfate saturation at pII 5.9. They were then dialyzed until free of sulfate, washed in 5 volumes of cold acetone, dried *in vacuo* over phosphorus pentoxide, ground, and dried again.

The solutions of the pseudoglobulins were freed by filtration of proteins precipitating at pH 5.9 and 33% ammonium sulfate saturation. They were fractionated 4 times each into 40%, pH 5.9, and 50%, pII 5.9, saturated ammonium sulfate-insoluble proteins, *e.g.*, into pseudoglobulins-I and pseudoglobulins-II, respectively. After dialyses until free of sulfates, they were isolated by cold acetone precipitation as described for the euglobulins. At the last centrifugation, all of the pseudoglobulins-I of normal dogs were distributed evenly among four 250 cc. centrifuge bottles, and one of these broke. The yield, therefore, is only an approximation. A correction for a loss of  $\frac{1}{4}$  of the actual yield has been applied in Table I.

Each protein was analyzed by previously described methods (1) for nitrogen, sulfur, lipids, carbohydrates, and the aforesaid amino acids.

### RESULTS

The amounts of euglobulins and pseudoglobulins-I and -II, as well as the total amounts of albumins isolated (Ref. 1, Table I), are given in Table I. The data indicate that during the course of the infection an increase in euglobulin content from 8.11% to 14.58% and 19.03% occurs in the normal, infected-A, and infected-B sera, respectively. The

<sup>2a</sup> We gratefully acknowledge the valuable cooperation we received from Mr. Robert J. Fitzgerald in the preparation of the pneumococci cultures, and the inoculations of the dogs.

TABLE I

*The Distribution of the Serum Protein Fractions in Each 100 g.  
of Isolated Total Dog Serum Proteins*

All values are corrected for moisture, ash, lipid, and carbohydrates

Protein	Type of dog serum		
	Normal	Infected-A	Infected-B
	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
Euglobulin	8.11	14.58	19.03
Pseudoglobulin-I	10.82 <sup>a</sup>	21.20	15.51
Pseudoglobulin-II	18.78	7.63	5.83
Total albumins <sup>b</sup>	62.32	56.61	59.64

<sup>a</sup> See text for applied correction.

<sup>b</sup> For details, see paper VIII of this series (1).

normal, infected-A, and infected-B pseudoglobulin-I concentrations also increase from 10.82% to 21.20% and 15.51%, respectively, while the pseudoglobulin-II concentrations decrease from 18.78% to 7.63% and 5.83%, respectively.

TABLE II

*Nitrogen, Sulfur, Carbohydrate, and Lipid Contents of Salt-Fractionated  
Euglobulins, Pseudoglobulins-I and Pseudoglobulins-II of Normal  
and of Type I Pneumococcal Infected Dog Sera*

	Euglobulins			Pseudoglobulin-I			Pseudoglobulin-II		
	Normal	Infected A	Infected B	Normal	Infected A	Infected B	Normal	Infected A	Infected B
	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
Nitrogen <sup>a</sup>	15.0	15.1	15.0	15.0	15.0	15.3	14.9	14.9	14.8
Sulfur <sup>a</sup>	1.1	1.1	1.3	1.3	1.1	1.3	1.1	1.1	1.2
Carbohydrate <sup>b</sup>	5.9	1.1	3.1	3.8	1.0	1.5	5.3	8.8	4.9
Lipid <sup>b</sup>	0.1	0.3	0.3	0.3	0.7	0.5	0.1	0.8	1.0

<sup>a</sup> Calculated on ash-, moisture-, carbohydrate-, and lipid-free basis.

<sup>b</sup> Calculated on ash- and moisture-free basis.

The nitrogen, protein-sulfur, carbohydrate, and lipid contents of the 3 globulin fractions are presented in Table II. The globulins of normal and abnormal dog sera have the same nitrogen contents of  $15.05 \pm 0.25\%$ , and the same protein sulfur concentrations of  $1.3 \pm 0.1\%$ .

The carbohydrate concentrations of the euglobulins decrease during the course of the infection from 5.9% to 4.1% and 3.4% for infected-A and -B, respectively. The corresponding pseudoglobulin-I values increase in their carbohydrate contents from 3.8% to 4.0% and 4.5%, respectively. The carbohydrate values of the pseudoglobulins-II are inconsistent, the mildly infected-A sera have the highest carbohydrate concentration, 8.8%, while those of the normal and the severely infected-B sera have the lower values, 5.3% and 4.9%, respectively.

The lipid concentrations of the 3 isolated euglobulins are the same, 0.3-0.4%. A slight rise above normal values occurs in the lipid contents of the pseudoglobulins-I, and a more pronounced one in case of the pseudoglobulins-II, namely, 0.3%, 0.7%, 0.5%, and 0.4%, 0.8%, and 1.0%, respectively.

The amino acid composition of the euglobulins, pseudoglobulins-I, and pseudoglobulins-II, calculated on an ash-, moisture-, carbohydrate-, and lipid-free basis, is given in Table III. For reasons previously discussed (1), and in accordance with Block and Bolling's suggestion (4), Table IV presents the same data calculated on a 16% protein

TABLE III

*Amino Acid Composition of Euglobulins, Pseudoglobulins-I and Pseudoglobulins-II of Normal Dog Serum, and of Dog Serum during Pneumococcal Pneumonia*

All values are calculated on an ash-, moisture-, carbohydrate-, and lipid-free basis

	Euglobulins			Pseudoglobulin-I			Pseudoglobulin-II		
	Normal	Infected A	Infected B	Normal	Infected A	Infected B	Normal	Infected A	Infected B
	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
Methionine	2.1	1.9	2.0	1.9	2.0	2.0	2.1	2.1	2.5
Cysteine	0.9	0.8	1.0	0.9	0.7	0.9	0.7	1.1	1.2
Cystine*	2.6	2.3	2.5	2.5	2.3	2.3	2.1	1.8	2.1
Cystine+cystine	3.5	3.1	3.5	3.4	3.0	3.2	2.8	2.9	3.3
Tyrosine	6.4	5.9	5.8	5.8	5.7	5.9	5.7	7.2	6.8
Tryptophan	3.1	2.7	2.9	3.0	2.7	2.7	2.5	2.7	2.3
Serine	13.3	12.0	12.9	11.1	12.8	13.8	13.9	14.3	13.0
Threonine	11.8	8.5	11.4	7.9	8.2	8.2	10.7	12.8	13.0
Hydroxyproline	0	0	0	0	0	0	0	0	0

\* The cystine values are calculated as the differences between the analytical cystine + cysteine and cysteine determinations.

TABLE IV

*Amino Acid Composition of Euglobulins, Pseudoglobulins-I and Pseudoglobulins-II of Normal Dog Serum, and of Dog Serum during Pneumococcal Pneumonia*

All values are calculated on a 16% protein nitrogen content from the data in Tables II and III

	$\pm\%$ <sup>a</sup> Error	Euglobulin			Pseudoglobulin-I			Pseudoglobulin-II		
		Normal	Infected A	Infected B	Normal	Infected A	Infected B	Normal	Infected A	Infected B
		<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
Methionine	6.6	2.2	2.0	2.1	2.0	2.1	2.1	2.3	2.6	2.7
Cysteine	3.0	0.9	0.8	1.1	0.9	0.8	0.95	0.8	1.2	1.3
Cystine	<sup>b</sup>	2.8	2.5	2.7	2.7	2.5	2.4	2.3	1.9	2.3
Cystine + cysteine	3.0	3.7	3.3	3.8	3.6	3.3	3.4	3.1	3.1	3.6
Tyrosine	2.0	6.8	6.3	6.2	6.2	6.1	6.2	6.1	7.7	7.4
Tryptophan	2.8	3.3	2.8	3.1	3.2	2.9	2.8	2.7	2.9	2.5
Serine	13.2	14.4	12.8	13.7	11.8	13.7	14.4	14.9	15.4	14.1
Threonine	13.8	12.6	9.0	12.2	8.4	8.7	8.6	11.5	13.8	14.1
Hydroxyproline	0	0	0	0	0	0	0	0	0	0

<sup>a</sup> The numbers given in this column represent maximum deviations from the mean of 10 analyses.

<sup>b</sup> The cystine values are calculated as the differences between the analytical cystine + cysteine and cysteine determinations.

nitrogen basis. The latter table will be used for a comparison of the data.

It is evident from Table IV that the methionine, cysteine, cystine, tryptophan, serine, threonine, and hydroxyproline concentrations of the euglobulins and pseudoglobulins-I, and the tyrosine values of the latter, are the same within the limits of accuracy of the methods, in the normal and infected sera. Apparently, a decrease from the normal tyrosine content occurred, however, in the euglobulins. The analyses for cystine, tryptophan, serine, and threonine in case of the pseudoglobulins-II are also the same, within the limits of the analytical procedures, for the normal and abnormal sera, but the normal values for methionine, cysteine, tyrosine, and perhaps cystine are lower than those from the pneumonic dogs. Whether these differences are real, we are not prepared to state, since the differences are only slightly greater than what one could expect from the assumed errors of the methods.



We feel that a new isolation and new analyses of the pseudoglobulins-II are necessary to establish with reasonable certainty that the observed differences are not artifacts. The methionine, cystine, and cysteine values, however, can be checked for accuracy by calculating the theoretical sulfur contents from the amino acid analyses, and comparing them to the total Pregl sulfur values. This is done in Table V. It is

TABLE V  
*Comparison of the Calculated Sulfur Values from Methionine, Cystine, and Cysteine Analyses of Normal and Pneumococcal Infected Euglobulins, Pseudoglobulins-I, and Pseudoglobulins-II with Pregl Sulfur Determinations*

	Euglobulins			Pseudoglobulins-I			Pseudoglobulins-II		
	Normal	Infected		Normal	Infected		Normal	Infected	
		A	B		A	B		A	B
		Per cent	Per cent		Per cent	Per cent		Per cent	Per cent
Sum of cysteine + cystine + methionine sulfur, calculated from amino acid analyses	1.37±0.06	1.24±0.06	1.36±0.06	1.31±0.06	1.23±0.06	1.28±0.06	1.20±0.06	1.29±0.05	1.42±0.07
Pregl sulfur analyses	1.4±0.3	1.4±0.3	1.3±0.3	1.3±0.3	1.4±0.3	1.3±0.3	1.4±0.3	1.4±0.3	1.2±0.3

evident that, even in the case of the pseudoglobulins-II, the calculated sulfur values from amino acid analyses check rather well the total sulfur determinations.

## DISCUSSION

The yield data presented in Table I show that during lobar pneumonia in dogs the total amounts of circulating serum globulins increase from 37.71% to 43.41% and 40.37%, respectively. This increase occurs in 2 of the 3 fractions, namely, in the euglobulins and in the pseudoglobulins-I, and is sufficiently great to compensate for a decrease in the pseudoglobulin-II fraction. Insofar as the total quantity of circulating globulins in health and disease is concerned, these data essentially confirm the many recorded observations from electrophoretic and salt fractionations.

A direct comparison of the quantitative aspects of the 3 individual fractions with published data is difficult for several reasons. In the first

place, it is generally agreed that none of the salt-fractionated serum proteins are homogeneous entities corresponding to one of the electrophoretic globulin fractions (5, 6). Secondly, published data deal primarily with human and some horse, cow, and swine sera, but not with dog sera. The consensus of opinion, however, seems to be (5, 6, 7) that, although each fraction is a mixture containing some of the  $\alpha$ -,  $\beta$ -, and  $\gamma$ -globulins, the euglobulin is fairly pure  $\gamma$ -globulin, pseudoglobulin-I is predominantly  $\beta$ -globulin and that the pseudoglobulin-II contains most of the  $\alpha$ -globulins along with some  $\beta$ - and  $\gamma$ -globulins.

Blix (8) published electrophoretic data on human sera during pneumonia. He observed concentration increases above normal values in the  $\alpha$  fractions. This would be chiefly our pseudoglobulin-II. However, we found increases in the euglobulins and pseudoglobulins-I, but not in the pseudoglobulins-II. Gutman *et al.* (9) reported the results of serum globulin salt fractionations of normal human sera and of sera during a variety of infections all of which caused hyperglobulinemia. Their data agree closely with ours in that the euglobulins and pseudoglobulins-I increase. Their pseudoglobulin-II fractions amounted to about 25% of the mean total globulin content and "in marked hyperglobulinemia this proportion often fell to less than 10%" (p. 771), which is entirely in agreement with our findings.

The close association of lipids and carbohydrates with the serum globulins is well established (see 10 for reviews). Blix, Tiselius, and Svensson (11), in an analysis of electrophoretically separated globulins of 3 normal human sera and one from a pneumonia patient, observed a large decrease in the cholesterol content in the  $\alpha$ -globulins and a small decrease in the  $\gamma$ -globulins, the  $\beta$ -globulins of the pneumonia patient not having been analyzed for cholesterol. A slight decrease of phospholipids in the  $\alpha$ -globulin was recorded, while the  $\beta$ - and  $\gamma$ -globulins increased in their phospholipid contents. These data are in fair agreement with ours, although ours are based only on the total ether-extractable lipids. The euglobulins ( $\gamma$ ?) decreased only slightly, from 0.4% to 0.3% and 0.3% for the two pneumonia sera. The pseudoglobulin-I ( $\beta$ ?) from pneumonia sera (0.7% and 0.5% respectively) contained more lipids than did the normal (0.3%), while the pneumonia pseudoglobulin-II ( $\alpha$ ?) lipid concentration increased markedly from a normal value of 0.4% to 0.8% and 1.0%.

These authors also report carbohydrate analyses of their electro-

phoretically separated globulins from 2 normal human sera and from one of a pneumonia patient. The carbohydrate content of the normal  $\alpha$ -globulins increased from 6.0% to 9.9% during pneumonia; that of the  $\beta$ -globulins decreased from normal values of 8.3% and 6.2% to 1.5%; and that of the  $\gamma$ -globulins remained about the same, namely, 3.6% and 3.0% for normal, and 3.7% for pneumonia serum. In contrast, we found in dogs a decrease from the normal euglobulin ( $\gamma$ ?) carbohydrate value of 5.9% to 4.1% and 3.4%, an increase in the pseudoglobulin-I fraction ( $\beta$ ?) from 3.8% to 4.0% and 4.5% and an undeterminable change in the pseudoglobulins-II ( $\alpha$ ?) from 5.3% to 8.8% and 4.9% in the pathological sera.

A direct comparison of our amino acid values with the literature is not possible since no amino acid values of dog serum globulins have been reported. Recently Brand *et al.* (12) published the results of amino acid analyses of electrophoretic, highly purified human  $\gamma$ -globulins of not more than 0.5% protein impurity, and of a  $\beta$ -globulin preparation with 11%  $\alpha$ - and 27%  $\gamma$ -globulins as impurities. A comparison of their human with our canine data is interesting and is summarized in Table VI. An inspection of this table indicates that the dog globulins contain more sulfur due to an increase in all 3 sulfur-containing amino acids.

TABLE VI

*Comparison of the Amino Acid Composition of 99.5% Homogeneous Human  $\gamma$ -Globulin and  $\beta$ -Globulins of 62% Purity (Brand, Kassell and Saidel (13)) with Normal Dog Euglobulins, Pseudoglobulins-I, and Pseudoglobulins-II*

	Human (13)		Dog		
	$\gamma$ -globulin	$\beta$ -globulin	Euglobulin	Pseudo-globulin-I	Pseudo-globulin-II
	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
Nitrogen	16.03	14.84	15.0	15.0	14.9
Sulfur	1.02	1.05	1.4	1.3	1.4
Methionine	1.06	1.54	2.1	1.9	2.1
Cysteine	0.7	0.2	0.9	0.9	0.7
Half-cystine	2.37	2.5	2.6	2.5	2.1
Cysteine + half-cystine	3.07	2.7	3.5	3.4	2.8
Tryptophan	2.86	2.06	3.1	3.0	2.5
Tyrosine	6.75	5.6	6.4	5.8	5.7
Serine	11.4	8.4	13.3	11.1	13.9
Threonine	8.36	7.26	11.8	7.9	10.7

The other amino acids fall within the same range in both sets of data, but individual absolute values are, at times, quite far apart.

#### ACKNOWLEDGMENT

We wish to express our appreciation to Miss Suzanne Spitz for her technical assistance during a part of this investigation.

#### SUMMARY

Pooled sera of normal dogs and of dogs during mild and severe experimental Type I pneumococcal pneumonia were fractionated with ammonium sulfate into euglobulins, pseudoglobulins-I, and pseudoglobulins-II.

The total amounts of isolated globulins in the sera of infected dogs were higher than in normal animals. This increase occurred in the euglobulin and pseudoglobulin-I fractions, and was large enough to compensate for a decrease in the total pseudoglobulins-II. The nitrogen and sulfur values of the 3 types of normal globulins and of those from infected dogs were the same,  $15.05 \pm 0.25\%$  nitrogen, and  $1.3 \pm 0.1\%$  sulfur. Decreased carbohydrate concentrations were found in the infected euglobulins, and increased amounts in the pseudoglobulins-I; the carbohydrate values of the pseudoglobulins-II were erratic, the mildly infected ones having higher and the severely infected ones lower than normal values. The lipids in the euglobulin decreased so slightly that the values are probably within the experimental error, but a definite increase in lipid concentration occurred in the infected pseudoglobulins-I, and a very pronounced one in the pseudoglobulins-II.

The methionine, cysteine, cystine, tryptophan, serine, threonine, and hydroxyproline concentrations of the euglobulins and pseudoglobulins-I, the tyrosine values of the latter, and the cystine, tryptophan, serine, and threonine concentrations of the pseudoglobulins-II remained essentially the same during the infection. Slightly decreased values were observed in the infected sera in case of tyrosine of the euglobulins, and increased values for the methionine, cysteine, tyrosine, and perhaps cystine of the pseudoglobulins-II.

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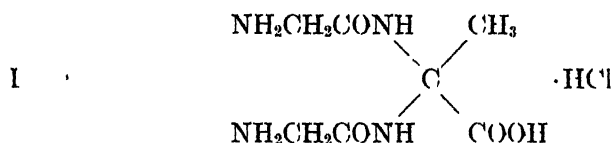
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## Enzymatic Splitting of $\alpha,\alpha$ -Diglycylaminopropionic Acid Hydrochloride

Sirs:

$\alpha,\alpha$ -Diglycylaminopropionic acid HCl (I) has been prepared by the condensation of chloroacetonitrile with pyruvic acid in concentrated sulfuric acid, followed by the amination of the resulting  $\alpha,\alpha$ -dichloroacetaminopropionic acid. The peptide has three ionizable groups, with  $pK_1 = 1.8$ ,  $pK_2 = 8.1$ , and  $pK_3 = 8.1$ . Melting point  $199^\circ\text{C}$ .



Unlike  $\alpha,\alpha$ -dichloroacetaminopropionic acid and  $\alpha,\alpha$ -diacetaminopropionic acid, I is readily split in descending order of activity by aqueous extracts of rat kidney, intestinal mucosa, pancreas, liver, spleen, muscle, and brain. Ammonia and pyruvic acid are produced in equimolar amounts, close to 1 mole of each product being the maximum formed per mole of substrate (Fig. 1). The reaction is optimal at pH 8.0, and at each pII value studied, from 6 to 10, the molar ratio of ammonia to pyruvate is close to 1.

I may be initially split (a) at one of the peptide bonds yielding glycine and  $\alpha$ -glycylamino- $\alpha$ -aminopropionic acid, followed by the spontaneous hydrolysis of the latter, unstable compound to products which include equimolar amounts of ammonia and pyruvate, or (b) at one of the bonds uniting a glycylamino residue with the tertiary carbon atom, yielding glycine amide and glycyldehydroalanine, and followed by the enzymatic hydrolysis of the dehydropeptide to equimolar amounts of ammonia and pyruvate (1). Glycine amide furnishes ammonia at a negligible rate.

The susceptibility of I to enzymatic attack is novel, and suggestive of the possibility of a condensation in tissues of amino acid amides with pyruvate to form diacylamino- $\alpha$ -aminopropionic acids as well as dehydropeptides (2).

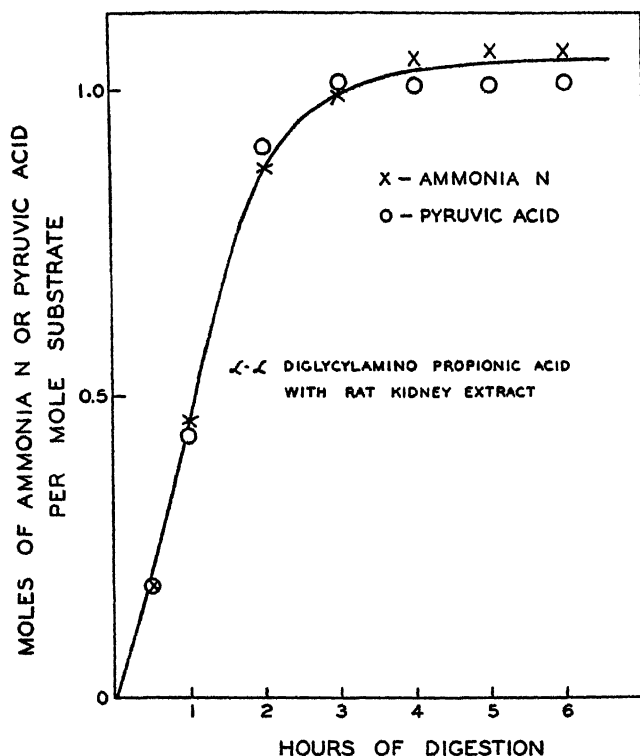


FIG. 1. Moles of ammonia N and pyruvic acid produced per mole of  $\alpha,\alpha$ -diglycylaminopropionic acid HCl in digests of aqueous, dialyzed rat kidney extracts as a function of the time of incubation. Digests consisted of 1 cc. extract, equivalent to 333 mg. fresh tissue, 2 cc. 0.15 borate buffer at pH 8.1, and 1 cc. of either water or substrate at 0.025 *M* concentration. Data corrected for extract blanks. Final pH 8.0. No decomposition products noted in digests with boiled extracts. Ammonia and pyruvate added to the extracts were recovered from 90–98%.

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National Cancer Institute,  
National Institute of Health,  
Bethesda, Maryland.

JOSE M. GONÇALVES  
JESSE P. GREENSTEIN

May 8, 1946.

## Some Spectral and Chemical Properties of Pyruvoylglycine

Sirs:

Aqueous solutions of pyruvoylglycine at  $\text{pH} < 10$  possess a characteristic absorption in the ultraviolet with maxima at 2400 Å and at 3400 Å (Fig. 1), and the peptide readily forms a crystalline 2,4-dinitrophenylhydrazone (m.p.  $242^\circ\text{C}$ .; Kjeldahl N found 12.7%, calculated 12.8%). On hydrolysis with boiling 2 *N* HCl, pyruvic acid and  $\alpha$ -amino nitrogen are nearly quantitatively recovered. The peptide possesses a  $\text{pK} = 3.3$  at  $25^\circ\text{C}$ . The corresponding  $\text{pK}$  of pyruvic acid is 2.5.

Aqueous solutions of pyruvoylglycine brought to  $\text{pH} > 10$  by addition of dilute NaOH lose irreversibly the characteristic absorption

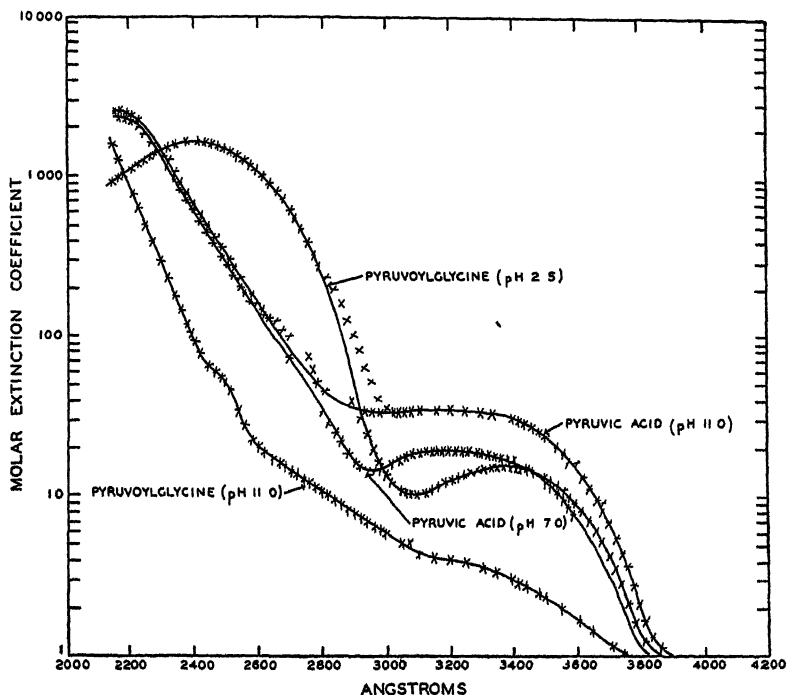
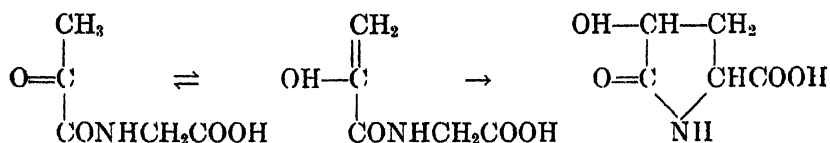


FIG. 1. Molar extinction coefficients, plotted on semilogarithm scale, of pyruvoylglycine and of sodium pyruvate as a function of the wavelength and of pH. Concentration of each component was  $5 \times 10^{-4} M$  measured between 2200 and 2900 Å, and  $2.5 \times 10^{-2} M$  measured above 2900 Å.



in the ultraviolet (Fig. 1), and the compound no longer forms a hydrazone. Acidification with HCl does not restore these properties. The compound possesses a  $pK = 3.3$ , and after isolation and purification has the same total nitrogen content as the original material before alkalization (9.5% N). On treatment with boiling 2 *N* HCl, neither pyruvic acid nor  $\alpha$ -amino nitrogen is recovered in the mixture. The possibility of an intramolecular rearrangement of the enolized form of pyruvoylglycine in alkali leading to ring closure and the formation of a  $\gamma$ -hydroxypyrrolidone carboxylic acid is suggested as explaining the phenomena noted:



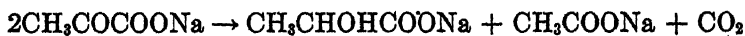
The characteristic absorption at 3300 Å of pyruvic acid disappears at pH > 10, but is restored when solutions of this compound are acidified, and the capacity to form the 2,4-dinitrophenylhydrazone is unaffected. The alkalization of pyruvic acid is thus reversible, that of pyruvoylglycine is irreversible.

National Cancer Institute,  
National Institute of Health,  
Bethesda, Maryland.  
May 29, 1947

MAURICE ERRERA  
JESSE P. GREENSTEIN

## Reversibility of Dismutation of Pyruvate

The anaerobic dissimilation of pyruvate by mammalian tissue and by certain bacteria has been described by Krebs and Johnson (1), Krebs (2) and Nelson and Werkman (3). The reaction appears to be an oxidation-reduction reaction involving two molecules of pyruvate as indicated by the following equation.



The purpose of this communication is to report the reversibility of this reaction using the stable isotope of carbon,  $\text{C}^{13}$ , as a tracer.

Cells of *Staph. aureus* were grown aerobically at pH 7.4 on a medium containing 1.0% peptone, 0.5% yeast extract, 0.5% beef extract, 0.5% sodium chloride, and 2.0% agar. After 20 hrs. incubation at 30°C., the cells were harvested and lyophilized. Cell-free extracts were prepared by grinding the freshly harvested cells with powdered glass as described by Kalnitsky *et al.* (4).

The exchange experiments were conducted anaerobically under oxygen-free nitrogen on a Warburg respirometer at a temperature of 30.4°C. The  $C^{13}$  content is expressed as the percentage in excess of the normal complement. All residual  $C^{13}O_2$  from the  $NaHC^{13}O_3$  was removed before decarboxylation of the pyruvate.

TABLE I  
*Exchange of  $C^{13}$  of  $NaHC^{13}O_3$  with C of Pyruvate Carboxyl*

Additions	Pyruvate remaining	$C^{13}$ per cent excess in carboxyl	$C^{13}O_2$ fixed
	<i>mM</i>		<i>mM</i> $\times 10^4$
<i>Cells</i>			
450 mg. lyophilized cells; 1.42 mM $NaHC^{13}O_3$ (3.29% $C^{13}$ excess)	0.178	0.00	0.0
Above additions plus 20 mg. A.T.P. (Pyruvate decarboxylated by yeast carboxylase)	0.351	0.07	2.8
<i>Cell-free Extract</i>			
15 ml. extract; 1.8 mM $NaHC^{13}O_3$ (5% $C^{13}$ excess)	0.398	0.06	2.4
Above additions plus 20 mg. A.T.P. (Pyruvate decarboxylated by $Ce(SO_4)_2$ )	0.334	0.19	6.3

Each cup contained 1.30 mM pyruvate; 1.5 mM phosphate buffer at pH 6.6, plus additions listed.

An excess of  $C^{13}O_2$  (Table I) was found in the pyruvate carboxyl in 3 of the 4 experiments, indicating a reversibility of this dismutation reaction. Adenosine triphosphate appears to accelerate reversibility, indicating that an energy-rich linkage is necessary. Increased exchange with ATP has been observed previously by Utter *et al.* (5) in the reversibility of the phosphoroclastic split of pyruvate and by Utter and Wood (6) in the fixation of  $C^{13}O_2$  in oxaloacetate by pigeon liver.

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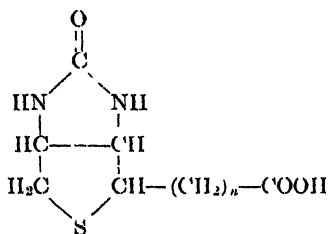
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Department of Bacteriology,  
Iowa State College,  
Ames, Iowa  
June 9, 1947

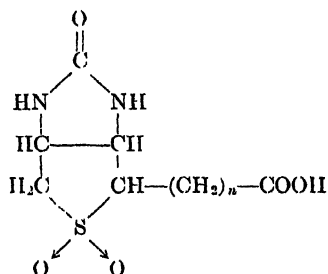
TORSTEN WIKÉN  
DEAN WATT  
A. G. C. WHITE  
C. H. WERKMAN

## Antibiotin Effect of Some Biotin Homologs

Biotin (I,  $n = 4$ ), its lower homolog nor-biotin (I,  $n = 3$ ), the higher homologs homobiotin (I,  $n = 5$ ), bis-homobiotin (I,  $n = 6$ ), and tris-homobiotin (I,  $n = 7$ ), have been synthesized by a new process which will be described elsewhere (1).



(I)



(II)

The new biotin homologs and three of the corresponding sulfones (II) were examined for antibiotin activity towards *Saccharomyces cerevisiae* 139 and *Lactobacillus casei* by the procedure of Dittmer and du Vigneaud (4), in, respectively, the media of Hertz (2) and Shull, Hutchings and Peterson (3). The values of the molar inhibition ratio (M.I.R.) for the present series are compared in the table with values for the most potent antibiotins reported in the literature, with the restriction that only determinations by the Dittmer-du Vigneaud procedure (4) were chosen; it is difficult to compare figures obtained by different inhibition measurements other than in a general way (which will be done in a full publication).

The following conclusions can be drawn from the table: 1. All the compounds of the present series are relatively potent antibiotics for both the bacterium and the yeast. 2. Homobiotin is the most potent of the group, and probably the most potent so far recorded, toward both these microorganisms. This is especially noteworthy because, of the antibiotics recorded in the literature, some, *e.g.*, desthiobiotin (4), biotin sulfone (4,9) and  $\gamma$ -(3,4-ureylene-cyclohexyl)-butyric acid (5), inhibit *L. casei* but not, appreciably, *S. cerevisiae*, while others, *e.g.*,  $\gamma$ -(2,3-ureylene-cyclohexyl)-butyric acid (5), inhibit the latter but not the former.

Homobiotin is also an antibiotic for *L. arabinosus*; in the range 1.0–0.6 m $\gamma$ , medium of Luckey *et al.* (7), the M.I.R. is 43,000.

The antibiotic activity of homobiotin is similar to the antioxybiotin effect of homooxybiotin towards *L. arabinosus* and *S. cerevisiae* described by Hofmann *et al.* (8).

*Antibiotin Effect of Biotin Homologs*

Compound	M P. corr	Molar inhibition ratio <sup>†</sup>	
		<i>Saccharomyces cerevisiae</i> 139	<i>Lactobacillus casei</i>
<i>d,l</i> -Norbiotin	230–233°	1,000	13,000
<i>d,l</i> -Homobiotin	220–221°	700	130
<i>d,l</i> -Bis-homobiotin	201–203°	30,000	7,000
<i>d,l</i> -Tris-homobiotin	216°	50,000	3,000
<i>d,l</i> -Homobiotin sulfone	260–261°	40,000	400
<i>d,l</i> -Bis-homobiotin sulfone	243–245°	60,000	8,000
<i>d,l</i> -Tris-homobiotin sulfone	244–246°	60,000	6,000

*Comparison with Some Potent Antibiotics Previously Reported*

<i>d</i> -Desthiobiotin (with biotin) (4)	Growth	9,000
<i>d,l</i> -Desthiobiotin (with biotin) (6)	Growth	17,000
<i>d,l</i> -Desthiobiotin (with O-heterobiotin) (10)	Growth	1,200**
<i>d</i> -Biotin sulfone (with biotin) (4)	Growth	280
<i>d,l</i> -Imidazolidonecaproic acid (with biotin) (4)	850,000	100,000

\* The molar inhibition ratio is defined (4) as the mols of antibiotic required to inhibit one mol of biotin when measured between the growth limits of 0.2 to 0.1 m $\gamma$  of biotin per tube. This procedure has the advantage of providing a relatively sharp endpoint.

\*\* Based on a modified definition of M.I.R., as measured by inhibition of O-heterobiotin from 1 to 0.5 m $\gamma$  per 10 cc.

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Research Laboratories of Hoffmann-La Roche, Inc.,  
Nutley, N. J.  
July 25, 1947.

M. W. GOLDBERG  
L. H. STERNBACH  
S. KAISER  
S. D. HEINEMAN  
J. SCHEINER  
S. H. RUBIN

## The Biotin Activity of Fat-Soluble Materials from Plasma

Sirs:

In connection with studies on the mode of action of biotin and certain of its analogues (1, 2), a search was undertaken in natural materials for substances other than biotin which exhibit biotin-like activity. In the course of these studies, the presence in lyophilized human plasma of an ether-soluble, neutral material capable of promoting growth of *Lactobacillus arabinosus* (3) in the absence of biotin was observed. Saponification of this material (4) yielded a biologically active, saponifiable fraction and a non-saponifiable fraction which was almost devoid of biological activity. Since it had already been shown (5) that certain unsaturated fatty acids stimulate growth of *Lactobacillus casei* in the absence of biotin (an observation which we were able to confirm with *Lactobacillus arabinosus*), it became of importance to establish whether the activity of our acid fraction was due entirely to its content of known unsaturated fatty acids. Work along these lines

was in progress when a paper appeared (6) in which it was stated that acid-hydrolyzed plasma contained a *non-saponifiable* ether-soluble substance which possessed biotin-like activity for *Lactobacillus casei*. These findings prompted the present communication.

It may be seen from Table I that the ether-soluble biotin-active sub-

TABLE I  
*Microbiological Activity of Ether-Soluble Materials from Human Plasma*

Fraction	Activity*
I. Ether-soluble, neutral	1.5
II. Saponifiable of (I)	5.0
III. Non-saponifiable of (I)	0.1
IV. Methyl esters of (II)	2.4
V. Oleic acid	5.0

\* Expressed as m $\mu$  of biotin/mg. of substance.

stance from plasma is acidic in nature, since invariably it accumulates in the saponifiable fraction (II). These experiments have been repeated several times with human as well as with beef plasma with identical results. Esterification with diazomethane of the biologically-active acid fraction (II), followed by distillation of the resulting methyl esters, yielded an ester fraction (IV) possessing high biological activity.

Since oleic acid has, on a weight basis, approximately the same activity as does our crude saponifiable material, it remains to be elucidated whether the activity of the plasma material is due solely to its content of this acid or whether it is due to another acid or acids with higher biological activity.

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Department of Chemistry,  
University of Pittsburgh, and the  
Institute of Pathology,  
Western Pennsylvania Hospital,  
Pittsburgh, Penna.  
June 9, 1947

KLAUS HOFMANN  
A. E. AXELROD

## Enzymatic Conversion of Ribose into Hexose-Monophosphate

Some time ago we reported the observation that ribose from adenosine, guanosine, and ribose-5-phosphate is converted by tissue enzymes into phosphoric acid esters which do not respond to the orcinol pentose reaction (1). We have now succeeded in isolating one of the reaction products, and we find it identical with Robison ester.

For the preparation, guanosine served as substrate. It was incubated with liver enzyme prepared as outlined earlier (1). Control experiments showed that no phosphate disappeared from the incubation mixture in absence of guanosine. The enzyme was free from glycogen. The mixture was deproteinized with trichloroacetic acid; the phosphoric acid esters were precipitated as lead salts, and divided into water-soluble and water-insoluble barium salts. Crude hexose-monophosphate, obtained from the former by alcohol precipitation, was purified according to Warburg and Christian (2), yielding the crystalline calcium salt. Twelve millimoles of guanosine gave 6.8 millimoles of crude product, from which 2.0 millimoles of pure ester were obtained. The product had the following analytical characteristics:

Elementary analysis:  $C_6H_{11}O_9PCa \cdot 2H_2O$  (MW 334.2).

Calculated: 21.53% C; 4.48% H; 9.30% P; 12.0% Ca;

Found: 21.35% C; 4.75% H; 9.80% P; 12.7% Ca.

Polarimetric determination: 83.7 mg. Ca-salt  $\cdot 2H_2O$  were dissolved in 0.65 ml. 1 *N* HCl + 6.35 ml. water.

$\alpha_D = +0.33^\circ$ , ( $l = 1$ );  $[\alpha]_D^{27} = +30.8^\circ$ .

Hypiodite reduction (3): 1.17 equivalents of iodine/*M* of ester.

Hydrolysis in 1 *N* HCl at 100°C.: After 30, 60, and 180 minutes: 10.7, 19.6, and 31.4% of the phosphoric acid split off.

An authentic specimen of Robison ester kindly furnished by Dr. E. Haas yielded identical results in hypiodite titrations, acid hydrolysis, fructose determination, and Seliwanoff test, according to the modification of Roe (4).

If our compound is tested with orcinol reagent, the absorption at 660  $m\mu$  is less than one-tenth of that obtained with an equimolar solution of pentose. This explains the negative result of the orcinol reaction in enzyme experiments reported earlier (1).

<sup>1</sup> C and H determination: Arlington Laboratories, Fairfax, Va.

For the conversion of pentose into hexose two explanations (5) seem possible: a direct extension of the five-carbon chain; or splitting of pentose into smaller units and recombination of two three-carbon compounds. We have reason to favor the latter explanation, although one might then expect hexose-diphosphate as the main product. However, our enzyme preparations contain phosphatase.

There have been some earlier indications of conversion of pentose into hexose. Ten years ago Z. Dische (6) briefly reported some data suggesting that ribose from adenosine may be converted into triose and hexose phosphate by incubation with hemolyzate. It is probable that his observations are analogous to our findings. A detailed account of the enzymatic mechanism and of the other reaction products will follow.

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*The University of Texas,  
M. D. Anderson Hospital for Cancer Research,  
Houston, Texas  
June 23, 1947*

M. J. WALDVOGEL  
F. SCHLENK

#### Erratum

In ARCHIVES OF BIOCHEMISTRY, Volume 11, Nos. 1/2, the formula on page 19 should read  $n(n+2)$  instead of  $n(n+1)$ .



## Book Reviews

**Reports of the Biochemical Research Foundation of the Franklin Institute. Vol. VIII. 1944-1945.** The Biochemical Research Foundation. Ca. 200 pp.

This book consists of three parts: reprints of original articles published by the research staff of the Biochemical Research Foundation, reprints of Notes from the Biochemical Research Foundation published in the Journal of the Franklin Institute, and an index.

Among the reprints there are 10 scientific articles and two reports by the Director. The titles and authors are as follows: (1) "The Biochemistry of *Vibrio cholerae*. I. Growth Methods," Richard W. Linton and Robert K. Jennings; (2) "The Biochemistry of *Vibrio cholerae*. II. The Influence of Environmental Factors on Growth," Robert K. Jennings and Richard W. Linton; (3) "The Synthesis of the Ethyl Esters of some 2-Oxo- and 2-Thio-1,2,3,4-tetrahydro-5-pyrimidinecarboxylic Acids," Donald W. McKinstry and Elizabeth H. Reading; (4) "The Study of Typhoid Antigens by Electrophoresis. I. Immunological Reactions," Richard W. Linton, Louis DeSpain Smith and Laura E. Krejci; (5) "The Biochemistry of *Vibrio cholerae*. III. Acid Regulation by Means of the Carbon Dioxide-Bicarbonate Buffering System," Robert K. Jennings and Richard W. Linton; (6) "The Ribonuclease Activity of *Pasteurella pestis* (Plague Bacillus)," Gladys E. Woodward; (7) "Progress of the Biochemical Research Foundation of the Franklin Institute 1943-1944," Ellice McDonald; (8) "Starch Digestion by *Vibrio cholerae* in Strongly Aerated Media," Robert K. Jennings; (9) "The Electrophoretic Analysis of Stored Liquid Human Plasmas," Laura E. Krejci, Lucile Sweeny and Edward B. Sanigar; (10) "Ribonucleinase. I. Manometric Determination of Ribonucleinase in Blood and Tissues of the Rat and the Rabbit," Charles A. Zittle and Elizabeth H. Reading; (11) "Ribonucleinase. II. Mononucleotides in Commercial Ribonucleic Acids and Their Effect on Ribonucleinase," Charles A. Zittle; (12) "Annual Report of the Biochemical Research Foundation of the Franklin Institute (1945)," Ellice McDonald.

The Biochemical Research Foundation Notes cover the period from January, 1944, to December, 1945. Included in them are abstracts of articles published in scientific journals, research reports intermediate in detail between usual technical articles and abstracts, and reports of the Director. These notes are reprinted from the *Journal of the Franklin Institute*.

The index is a combined author-subject index covering the first eight volumes of "Reports." It lists titles, authors, original place of publication, and the volume number of "Reports of the Biochemical Research Foundation" in which the collected reprints are bound. Two hundred twelve reprints published between 1930 and 1946 and Notes published between 1938 and 1946 are indexed.

MAX A. LAUFFER, Pittsburgh, Pa.

**Advances in Carbohydrate Chemistry, Vol. II.** Edited by W. W. PIGMAN AND M. L. WOLFROM. Academic Press Inc., New York, N. Y., 1946. xiv + 323 pp. Price \$6.60.

As was the case in Volume I, pure structural chemistry competes with the biochemistry of the carbohydrates for the dominant position in Volume II. A descriptive essay (43 pages) by J. H. Haskins on "Cellulose Ethers of Industrial Significance" is also included.

The volume opens with C. S. Hudson's vivid account (36 pp.) of the history, occurrence and structural chemistry of the non-reducing trisaccharide melezitose, one of whose glycosidic configurations is still in doubt. The related chemistry of the reducing disaccharide turanose is also carefully discussed. A complete treatment (37 pp.) of the "Chemistry of Anhydro Sugars," by S. Peat, includes valuable tables giving physical constants and literature references for all definitely characterized representatives containing three to seven membered anhydro rings. A similarly thorough and competent review of the "Analogues of Ascorbic Acid" (24 pp.), by F. Smith, includes critical assessments of alternative syntheses, discussions of desoxy, amino and other derivatives and of physiological properties. The contributions, by R. Lespieau, "Synthesis of Hexitols and Pentitols from Unsaturated Polyhydric Alcohols" (11 pp.), and by E. L. Hirst and J. K. N. Jones, "The Chemistry of Pectic Materials" (16 pp.), although shorter, adequately cover the subject matter. A review of "Polyfructosans and Diffructose Anhydrides" (24 pp.) by Emma J. McDonald (bacterial levans being omitted) completes the portion of the book dealing mainly with structural chemistry.

More than one third of the book (113 pp.) is devoted to three long, careful articles discussing the "Interruption of Carbohydrate and Fat Metabolism," the "Chemistry of Mucopolysaccharides and Mucoproteins," and "Bacterial Polysaccharides" (including the levans). The first of these articles, by H. J. Deuel and Margaret G. Morehouse, makes it clear that carbohydrate is readily transformed to fat in both plants and animals and that the reverse change normally occurs in plants. After critically discussing a great mass of contradictory and indecisive experimental evidence (247 references), the authors express the tentative opinion that higher animals are unable to promote a fatty acid  $\rightarrow$  carbohydrate change. The second article, by M. Stacey, opens with tables that somewhat modify Levene's classification of the subject matter into mucopolysaccharides (with a low but biologically significant protein content), mucoproteins (with a relatively high protein content and always containing hexosamines) and mucolipids. What little is known of the structural chemistry of these complex and often ill-defined entities is then summarized, together with a brief account of the chemistry of basic units like glucosamine and the uronic acids. The review discusses such substances as hyaluronic acid, chondroitin, pneumococci specific polysaccharides, carbohydrates characteristic of various blood groups, etc., always with reference as to their probable biochemical or physiological role. The third article, by T. H. Evans and the late H. Hibbert, is in similar vein and its title accurately defines its scope.

Volume II of the *Advances* is international in character, with contributions from Britain, Canada and France as well as the United States. Another pleasing feature is the very good photograph of the late Dr. R. Max Goepff, Jr., which serves as the frontispiece. The volume is physically very satisfactory and the reviewer noted only one error in it that might cause confusion and that is the reference on p. 208 to the obsolete claim that all undegraded celluloses contain 0.28% of carboxyl groups.

CLIFFORD B. PURVES, Montreal, Canada.

**Actions of Radiations on Living Cells.** By D. E. Lea, Proplit Student of the Royal College of Surgeons, Cambridge University Press and Macmillan Company, New York, N. Y., 1947. xii + 402 pp. Price \$4.50.

This is an excellent book, critically and competently written. Moreover, it could scarcely have come at a more opportune time, for the biological effects of radiation have assumed, since August, 1945, a potential significance far beyond that envisioned in the many previous years of academic research or clinical practice.

Among other distinctive features, the point of view throughout is fundamental and quantitative. In the first two chapters the basic physical principles of excitation, radiation, ionization, absorption and dissipation of energy, and chemical effects of ionizing radiations are concisely set forth, accompanied by simplified but adequate mathematical formulations. A short, general discussion is given of the possible modes of biological action of ionizing radiations. Chapter 3 deals with the important aspects of the target theory with which the remaining six chapters of the book are largely concerned in relation to specific problems: the inactivation of viruses by radiation; genetic effects, including gene mutation as well as structural and physiological changes in chromosomes; delayed cell division; lethal effects; and the mechanisms involved, insofar as available evidence is sufficient to conclude or postulate.

In addition to 61 well constructed graphical figures or diagrams, and 4 plates, a total of 83 tables of data provide valuable calculations derived from theory and observation. They should prove useful to anyone actively engaged in radiation research in biology. These tables, as well as the clearly expounded physical theory, should be particularly useful to biologists just entering the field of radiation studies, or to those accustomed to consider the phenomena from a largely qualitative point of view. Conversely, for the physicist and others, the biological basis, including the specialized methods and terminology of genetics, is tersely described, beginning with elementary principles and moving swiftly to complex problems.

It was the author's expressed purpose not to survey the whole field, but rather to present a somewhat "detailed discussion of the mechanism of those actions of radiations which are sufficiently well understood for such a treatment to be profitable at the present time." Thus, some of the well known but essentially qualitative recent radiation studies are not included. The material that is discussed, however, has been chosen thoughtfully and with good judgment. Most of the some 400 references are to publications within the past decade. This book is highly recommended to investigators and students interested in the fundamental aspects not only of the biological actions of radiations, but also of genetics, the phenomena of growth and integrity of cells, and medical research.

FRANK H. JOHNSON, Princeton University.

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